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Observations on the Microbiological Determination of Riboflavin in Blood

By ENID A. M. BRADFORD and H. COKE, *Charterhouse Rheumatism Clinic, London, W.* 1

(*Received* 5 *January* 1945)

The present investigation was undertaken to devise a clinically useful method for the microbiological determination of the riboflavin content of the blood in various rheumatic diseases, and to elucidate certain factors which might influence the results.

The microbiological method of Strong, Feeney, Moore & Parsons (1941) involves the use of whole haemolyzed blood, which, on being autoclaved, produces a large amount of red-brown, fibrinous solid matter. This solid introduces uncontrollable variations into the final result. Further, the medium must be freshly prepared and it is difficult to deal with samples in an out-patient clinic if they cannot be collected into a working group over a period of 24 hr. Strong *et al.* (1941) found that their results were concordant when using tubes containing less than 0·2 ml. blood, but were inconsistent with larger volumes of blood. We find it more satisfactory to make an acid extract of blood, and so to remove a large proportion of the solid matter before the material is added to the assay tubes. The acid extract is stable and can be kept overnight, which greatly facilitates routine practice.

EXPERIMENTAL

Reagents required. Pure culture of *Lactobacillus casei* ϵ; 0·1 N-HCl; 0·1 N-NaOH; 0·02 N-acetic acid; sterile saline (0·85% (w/v)); Indicators (British Drug Houses Ltd.): (*a*) bromo-thymol blue; (*b*) bromo-thymol blue triple strength (capillator); (*c*) bromo-cresol green (capillator); culture medium as described by Snell & Strong (1939); stock riboflavin solution (100 μg./ml.) in 0·02 N-acetic acid prepared every 2 or 3 weeks, and stored under toluene in dark brown glass-stoppered bottles; Difco Yeast Extract (Digestive Ferments Co., Detroit); basic lead acetate ('Analar', British Drug Houses Ltd.); ammonia solution 3·1% (w/v); cellulose wadding (B.P. quality, Boots Pure Drug Co. Ltd.).

Organism used in assay. We experienced some irregularity at first with the culture obtained from the National Collection of Type Cultures (N.C.T.C., Lister Institute, Elstree) of *Lactobacillus casei* ϵ (Serial no. 7649 from the American Type Culture Collection). The culture from Elstree, differentiated on Crowe's (1927) chocolate medium, gave three definite morphological variants with differing responses to riboflavin (Fig. 1). Another culture from Mr S. A. Price (Vitamins Ltd.), also from the N.C.T.C. originally, gave the same three variants (Fig. 1). A culture from Dr J. G. Davis (Shinfield), which was found to be pure, has been used in all our work.

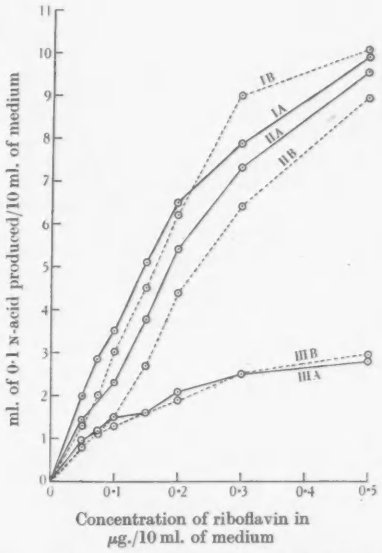

Fig. 1. Growth response of different morphological variants in cultures of *L. casei* ϵ. Curves IA, IIA, IIIA. strains isolated from Lister Institute, Elstree culture, showing differences in acid production with same concentration of riboflavin. Curves IB, IIB, IIIB: similar to series IA, IIA, IIIA, but repeated at a later date with another specimen of Lister Institute, Elstree culture.

The previous nutritional history of the organism also affected the accuracy of the results. At first we carried the organism by daily subcultures into the assay medium containing 1·0 μg. of riboflavin/10 ml. of medium (cf. Snell & Strong, 1939), returning to the stab culture once a week. We were puzzled by the initial 'kick' in the standard curve, as often a bigger response was given by 0·05 μg. than by 0·075 μg. of riboflavin, and differentiation between these small quantities was difficult. These results were far more regular when the inoculating emulsion was prepared from a single growth in liquid

medium, inoculated 24 hr. previously from the stab culture. In a stab culture several days old the organism probably tends to become deficient in riboflavin so that on subculturing in the assay tubes its response will be more nearly linear. On the other hand, in a liquid medium rich in riboflavin, some storage of the vitamin may occur either within the bacterial body or by adsorption on the cell surface. The effect produced is a much more vigorous growth by repeated subcultures in liquid medium, and the acid production is much more erratic. Fig. 2 shows the typical effect obtained with 'deficient' and 'replete' cultures.

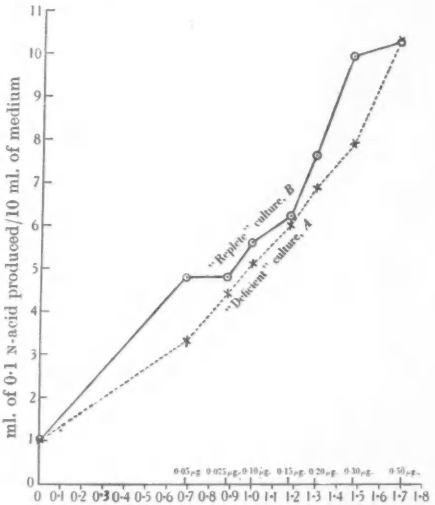
ml. of 0·1 N-acid produced/10 ml. of medium
"Replete" culture, B
"Deficient" culture, A
0·05 μg. 0·075 μg. 0·10 μg. 0·15 μg. 0·20 μg. 0·30 μg. 0·50 μg.
Log concentration of riboflavin/10 ml. of medium

Fig. 2. Effect of previous nutritional conditions on the response of *L. casei* ϵ to riboflavin. Curve *A*. 'Deficient' culture, i.e. 24 hr. broth culture direct from a stab culture 4 or 5 days old. Curve *B*. 'Replete' culture, i.e. 24 hr. broth culture after subculturing 4 days in succession on a broth rich in riboflavin (1 μg./10 ml.).

Preparation of medium. The alkalized photolyzed peptone (Snell & Strong, 1939) keeps well under toluene and can be made in bulk and used as required. The yeast supplement must be freshly prepared (from Difco yeast extract) for every batch and used within 24 hr. It appears to contain a labile factor which has a marked effect on the acid production, this being revealed as a central depression in the theoretically linear logarithmic curve. Fig. 3 shows a typical standard curve using a yeast extract several weeks old, compared with a similar curve obtained with fresh yeast extract 24 hr. old.

The yeast supplement is prepared as follows: 2·5 g. Difco yeast extract in 12·5 ml. distilled water are mixed with 3·75 g. basic lead acetate in 12·5 ml. distilled water and the pH adjusted to 10 by a few drops of ammonia (3·1% w/v). It is easy to judge the pH at which maximum precipitation of the solids occurs. The mixture is then filtered through a thin layer of cellulose wadding and acidified with enough glacial acetic acid to ensure complete precipitation

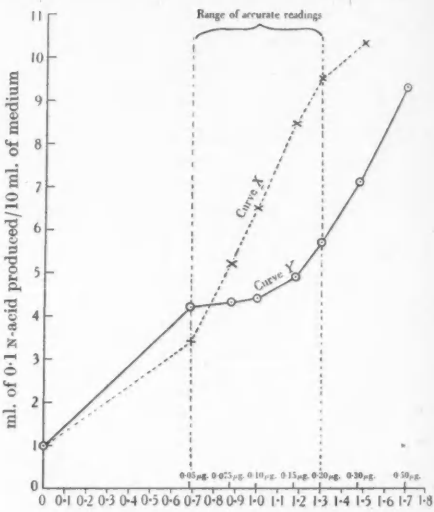
Range of accurate readings
ml. of 0·1 N-acid produced/10 ml. of medium
Curve X
Curve Y
0·05 μg. 0·075 μg. 0·10 μg. 0·15 μg. 0·20 μg. 0·30 μg. 0·50 μg.
Log concentration of riboflavin/10 ml. of medium

Fig. 3. Effect of ageing of riboflavin-free yeast supplement used in preparation of basal medium on the subsequent response of *L. casei* ϵ to riboflavin. Curve *X*. Deficient culture grown on medium containing freshly prepared yeast supplement. Curve *Y*. Deficient culture grown on medium containing old yeast supplement stored in the refrigerator for several weeks.

of the lead sulphide by H_2S and to prevent frothing during the passage of the gas. The lead sulphide is filtered off through cellulose wadding and the filtrate tested by bubbling H_2S until no further precipitation of lead occurs. The final solution is filtered through filter paper (Whatman no. 40) and boiled rapidly for 1–2 min. to remove H_2S. When cool, the solution is neutralized to pH 6·8 with N-NaOH, made up to 25 ml. and immediately placed in the refrigerator until required. Neutralization of the extract gave more consistent blank values in the assay tubes and the standard curve was more nearly linear. 5 ml. of this solution are required for every 250 ml. of concentrated medium.

Salt solutions are the same as those recommended by Snell & Strong (1939) but are made up in freshly boiled glass-distilled water and stored under toluene at room temperature.

Autoclaving. Precautions should be taken to prevent wetting of plugs during autoclaving. Unless the time and temperature are rigidly controlled, the medium darkens, which causes difficulty in reading the end-point in the final titration.

Preparation of acid extract of blood. Various concentrations of hydrochloric acid were tried in order to determine the optimum concentration for the extraction of riboflavin from blood.

The riboflavin content of the same sample determined direct by dilution with water only was used as a standard and the riboflavin determined on 0·25 ml. of blood, all other conditions being similar.

Table 1. *Effect of concentration of* HCl *on extraction of riboflavin*

(Results are the average of three replicates for each concentration of HCl.)

Sample	0·1 N-HCl in 10 ml. of extract (ml.)	Riboflavin content of whole blood (μg./100 ml.)	Percentage* of riboflavin extracted
X	4·0	35·2	100·0
	3·5	34·4	97·7
	3·0	28·8	81·8
	2·5	24·8	70·4
	2·0	22·8	64·7
	1·5	20·8	59·0
	1·0	16·4	46·5
	0·5	14·8	42·0
Y	4·0	24·0	100·0
	N-HCl in 10 ml. of extract (ml.)		
Y	4·0	22·0	91·6
	3·0	20·0	83·3
	2·0	16·0	66·6
	1·0	17·0	70·8
	0·75	22·8	95·0
	0·5	20·8	86·6
	0·25	26·0	108·3
	0·1	17·6	73·3
	Nil	20·0	83·3

* The results for percentage extraction were calculated on the 4·0 ml. of 0·1 N-HCl figures.

The results indicate that no advantage is obtained by increasing the concentration beyond 4 ml. of 0·1 N-HCl in 10 ml. extract (Table 1). The acid extract is adjusted to pH 6·8 by addition of NaOH. The addition of the neutralized extract to the medium might cause inhibition of growth on account of the increased concentration of sodium ions. That possibility was ruled out when an excess of either sodium chloride or sodium nitrate was found to have little effect upon growth.

The acid extract of blood thus prepared was checked against the original method of Strong *et al.* (1941) and found to give consistent results within the limits of experimental error (Table 2).

These results, obtained at the beginning of the work, indicate good agreement on the same levels for the whole blood and acid extract. The variation observed at different levels is dealt with later on and is apparently due to another factor added with the blood. Taken from patients with pathological conditions these samples of blood might be expected to show other variables.

Table 2. *Riboflavin content as determined on whole blood compared with that determined on the acid extract from the same sample*

	Fresh whole blood		Acid extract of blood	
Date and Subject	Blood (ml.)	Riboflavin content (μg./100 ml.)	Blood (ml.)	Riboflavin content of blood (μg./100 ml.)
4. vi. 43 Subject R	0·1	30·0	0·15	33·3
	0·2	32·5	0·20	32·5
	Mean	31·2	0·30	28·5
			Mean	31·4
4. vi. 43 Subject L	0·15	41·0	0·15	40·0
	0·20	35·0	0·20	36·0
	Mean	38·0	Mean	38·0
22. vi. 43 Subject J (saturated with riboflavin)	0·10	40·0	0·10	40·0
	0·15	36·6	0·15	36·0
	Mean	38·3	Mean	38·0

Standardization of volume of inoculum. As a result of parallel unpublished work by one of us (H. C.) on the population of cultures of *Staphylococcus aureus* it was apparent that the curves obtained in these microbiological assays are not 'growth curves' in the sense that measurement of the growth of animals is used in biological assay work. The acid production after 72 hr. incubation on any level of riboflavin is a measure of the maximum population supportable in the medium on that level of riboflavin. At lesser intervals of time the population may not have reached the stable maximum, in which case neither the acid production nor the opacity will bear a direct linear relationship to the concentration of riboflavin originally present in the tube. It has also been observed that the maximum stable population of *Staph. aureus* in a simple peptone medium can vary in direct relationship to the concentration of added food factors, e.g. glucose (cf. Hinshelwood, 1944). Hence to obtain true relative readings of riboflavin content in the Snell & Strong basal medium the following conditions must be assured: (*a*) time must be allowed for the population to reach a stable maximum; (*b*) the size of the original inoculum must be standardized; (*c*) the culture should be in a certain relatively deficient state. Hence we standardize the size of the drop. The weekly variations in growth response may be standardized by using a saline suspension of known opacity measured on the Spekker photoelectric absorptiometer, or by visual comparison with a standard suspension sealed in a centrifuge tube.

It is necessary to shake the tubes at least once during the 72 hr. incubation period in order to break

up bacterial clumps and thus ensure a more even rate of propagation. In measuring the rate of growth by opacity in less than 72 hr., as recommended in some papers, these factors are very important.

Poole & Hinshelwood (1940) and Hinshelwood (1944) were able to observe in cultures of *B. lactis aerogenes* four stages in the development of the stable maximum population: (*a*) lag-phase reaction, which is concerned with slow building up of some substance necessary for cell division; (*b*) actual processes involved in cell division; (*c*) metabolic reactions which go on even when active division has ceased; (*d*) lethal processes in which the actual death of the cell is accelerated. The growth-rate and stable maximum population which a given medium will support vary in a remarkably parallel manner, and growth of population follows closely the logarithmic law $dn/dt = kn$. At the end of the logarithmic phase growth falls off abruptly. It is therefore important in microbiological assays to select a time for titration at which the bacterial population is at a maximum and when the third phase of fermentation is complete. This occurs before the final or death phase of the culture is reached. In experiments on gas formation, Poole & Hinshelwood (1940) found no measurable amount of gas was collected until the bacteria had attained their stationary phase of growth, when the gas volume (presumably the fermentation stage) increased linearly with time, then abruptly ceased to increase.

The stationary phase is not due to a simple balancing of death-rate and division-rate but depends on actual cessation of division. This may be due to a decrease in the amount of a metabolite necessary for the fermentation reactions, e.g. riboflavin. The authors quoted above were working on the inhibitory action of disinfectants, e.g. phenol, mercuric chloride, etc. which had a toxic action. It is possible that the inhibitory substances which we found in blood may act at any one of the four phases of growth by immobilizing one of the essential metabolites in the development of *L. casei* ε.

RIBOFLAVIN IN BLOOD OF RHEUMATIC PATIENTS

Procedure

Preparation of extract. 1 ml. venous blood is mixed, immediately after withdrawal, with 4 ml. 0·1 N-HCl in a conical centrifuge tube which is then closed with a waxed cork and inverted several times to mix its contents. The mixture turns dark brown in colour. A cotton-wool plug is substituted for the cork, care being taken to prevent loss of liquid on sides of cork and tube. The tube, which is placed in a tall 400 ml. beaker without lip and protected against moisture, is autoclaved (at a pressure of 15 lb./sq.in.) for 15 min. After autoclaving the tube is immediately placed in a dark cupboard to cool. The extract is stable for at least 24 hr. whereas the unheated acid solution deteriorates overnight. When the assay is begun the contents of the tube are neutralized with 0·4 ml. of N-NaOH, mixed and back-titrated to pH 4·5 with a few drops of 0·02 N-acetic acid (bromo-cresol green as indicator) and centrifuged. It is convenient to use tubes of equal weight for a series of tests. The supernatant liquor is transferred to a standard 10 ml. measuring flask. 2 ml. of glass-distilled water are added to the sediment and the mixture stirred by means of a fine sealed capillary tube and again centrifuged. The washing process is repeated with 2 ml. water, and the combined washings added to the 10 ml. flask. This extract is adjusted to pH 6·7 with a drop or two of 0·1 N-NaOH before making up to 10 ml. The final solution may be centrifuged again if necessary.

The isoelectric point of most of the blood proteins is near pH 4·5. Precipitation at this pH ensures that a relatively small proportion of coagulable matter is carried through into the assay tubes.

Riboflavin solutions for standard curve. The stock solution of riboflavin is diluted with glass-distilled water to contain 0·1 μg./ml. for all subsequent measurements, so that the smallest volume measured is 0·5 ml. in the standard curve.

Preparation of assay tubes. A standard curve is prepared with each batch of tests. Graded quantities of standard riboflavin solution are added to the 5 ml. of medium, and the volume is made up to 10 ml. by running freshly distilled water in from a burette with the mouth of the test-tube held against the tip of the burette and the test-tube rotated rapidly in the left hand. To avoid contamination of the contents of one tube with that of another we always inoculate and measure volumes to the tubes in order, starting with the lowest concentration.

The neutralized acid extract of blood is then added to the assay tubes in the same manner, and portions of 2 and 2·5 ml. in duplicate (equivalent respectively to 0·2 and 0·25 ml. of blood) are satisfactory. With less than 0·2 ml. of blood, the quantity of acid produced was below the lowest measurable quantity of riboflavin on the standard curve. The total volume is made up to 10 ml. as for the standard curve. The assay tubes and the standard tubes are autoclaved for 15 min. at a pressure of 15 lb./sq.in. at the same time and are then ready for inoculation. All measurements are done in a darkened room (diffused daylight) and all tubes, etc. containing riboflavin are protected from light at all stages.

Inoculation. The organism is carried in stab cultures in yeast-water-agar containing 1% of glucose; this is quite satisfactory if a 24 hr. culture in liquid medium is taken direct from stab. After centrifuging for 10 min. the bacterial cells are emulsified with 10 ml. sterile saline and may be checked for opacity on the Spekker photoelectric absorptiometer. To avoid standardization of capillary pipettes a sterile 1 ml. syringe fitted with a no. 10 (Allen & Hanbury Ltd.) needle about 2·5 in. long, from which the oblique tip has been cut, is satisfactory when a number of tubes are to be inoculated. Inoculation is accomplished by holding the tube in a vertical position with the needle at right angles to the axis of the tube and then releasing one drop rapidly. If alternate drops are discarded there is little danger of contamination from the outside air. The contents of the tubes are well and carefully mixed by agitation and rotation.

Incubation. When inoculated the tubes are incubated at 37° for 72 hr. At least once during the incubation period

and preferably after each 24 hr. the tubes are removed and mixed carefully and thoroughly to break up bacterial clumps.

Titration. If there is an appreciable amount of coagulum in the tubes containing blood it may be rapidly removed by filtering through a 1 in. square of cellulose wadding split to a half or a third of its thickness. These squares are washed with distilled water whilst supported in 1·5 in. funnels and the solution is filtered rapidly into 50 ml. hard glass Erlenmeyer flasks. The tube and filter pad are washed twice with 5 ml. of freshly distilled water and the excess moisture expressed from the filter pad by rolling and pressing with a fine glass rod. The contents of the assay tubes thus diluted to 20 ml. with washings are titrated against 0·1 N-NaOH with bromo-thymol blue as internal indicator. In the first instance 10 drops of indicator are added and then as titration proceeds 1 drop of indicator is added for every 1 ml. of alkali used. This keeps the intensity of the colour constant with increasing dilution and gives greater accuracy at the higher levels of riboflavin.

Examination of results. The acid production, plotted against the log of the concentration of riboflavin in the standard tubes, gives a straight line and a truer picture of the bacterial population than the ordinary standard curves of Snell & Strong (1939).

Accuracy of the method. Over a period of 11 months 43 standard curves have been determined. The correlation coefficient r, determined from the mean values of the 43 estimations for each standard concentration of riboflavin, shows a value $= +0{\cdot}85$, which suggests a significant correlation. During this time the refinements in technique here described were evolved, so that the correlation coefficient taken on the same basis for the last fifteen curves may be expected to be a more accurate assessment of this technique, and shows a figure $r = +0{\cdot}98$.

The assay tubes for normal blood usually give a reading equivalent to an amount of 0·075 μg. We derive the figures given in Table 3 from the series of 43 estimations of this amount.

Table 3. *Accuracy of the method*

	Mean value for tube containing 0·075 μg. riboflavin	Standard deviation of mean	Standard error of mean	Standard error as % of total
Total from 43 curves	3·88	0·80	0·122	3·14
Last 15 curves	4·42	0·73	0·188	4·25

The small number of readings obtained with a single specimen of blood and the effect of the unknown quantities and qualities of inhibitory substances does not permit a satisfactory statistical analysis of the accuracy of the method. We would estimate roughly from practical experience that the results lie within the range $= \pm 2$ μg./100 ml. of blood ($\pm 0{\cdot}005$ μg. in the volume estimated). With an average reading of 25 μg./100 ml. this is equivalent to $\pm 8\%$, the error becoming greater with smaller totals and less with greater total contents. The figure thus obtained is about twice the standard error (Table 3), as might be expected.

Results

The above method was devised to obtain information about the riboflavin concentration in the blood in chronic rheumatic disease. The results here reported were measured in duplicate on 0·2 and 0·25 ml. of whole blood; eight normal subjects gave an average of 21·2 μg./100 ml. One normal individual was tested on four occasions and gave the following results:

1943	μg./100 ml.
May	31·0
June	35·0
September	13·1
September (1 week later)	13·5

This shows the likely variations to be expected over a period of war-time diet.

From 56 cases of chronic rheumatic disease the results, given in Table 4, were obtained.

Table 4. *Riboflavin content of rheumatic blood*

Riboflavin content (μg./100 ml.)	10–30	31–50	Above 50
No. of cases	40	13	3
Mean riboflavin content (μg./100 ml.)	21·3	43·7	76·3

Relation between riboflavin content and differential sedimentation test (D.S.T.) *of rheumatic blood*

D.S.T.* (%)	1–10	11–20	21–30	31–40	41–90
No. of cases	12	1	2	1	3
Mean riboflavin content (μg./100 ml.)	23·0	(33·5)	22·5	25	22

* The differential sedimentation test was carried out by the method of Coke (1937).

There is no striking difference between the riboflavin contents of normal and of rheumatic bloods and it is difficult to see any significant correlation between the riboflavin content of the blood and any other factor associated with the rheumatic condition, either of the clinical diagnostic type or of the nature of a laboratory assessment such as a differential sedimentation test (Coke, 1937) of the blood. Of particular interest was a patient with acute iridocyclitis who had been saturated with riboflavin. He was excreting 9·5 mg. of riboflavin/100 ml. of urine; his blood riboflavin was only 42 μg./100 ml. From the clinical aspect one can record only the impression that the blood riboflavin may possibly be related to the patient's resistance or reactive capacity and thus may show a similarity to the results reported by Cottingham & Mills (1943).

SOME FACTORS AFFECTING THE MICROBIOLOGICAL ASSAY OF RIBOFLAVIN

Inhibitory factors in blood. To determine the accuracy of the method recovery experiments were carried out. A pooled sample of blood from five

patients was freed from riboflavin by the following method:

5 ml. of the pooled blood were diluted with 20 ml. of 0·1 N-HCl and mixed with 5 ml. of a solution containing 1·08 g. of NaOH. (0·08 g. of NaOH is required to neutralize the 20 ml. of 0·1 N-HCl and the resulting solution therefore contained 4% NaOH.) The 30 ml. of alkalinized blood were placed in a beaker of 2¼ in. diameter and irradiated for 10 min. at a distance of 20 in. from an ultra-violet lamp (Hanovia Ltd.) by direct rays. The solution was transferred to a 50 ml. flask, neutralized with 0·96 ml. of glacial acetic acid, the pH adjusted to 6·7–6·8 and the volume made up to 50 ml.

Table 5. *Effect of alkalinized, photolyzed blood on recovery of added riboflavin*

Date	Riboflavin added to medium (μg./10 ml.)	Blood added to medium (ml./10 ml.)	ml. of 0·1 N-acid formed*	Riboflavin found (μg./10 ml.)	Loss (%)
8. x. 43	0·1	0·05	6·95	0·12	(gain 0·02)†
		0·10	6·6	0·10	Nil
		0·15	3·4	0·03	67
		0·20	Nil	Nil	100
		0·25	Nil	Nil	100
		0·30	Nil	Nil	100
8. x. 43	0·2	0·05	8·7	0·22	(gain 0·02)†
		0·10	8·05	0·18	7
		0·15	2·05	0·16	17
		0·20	Nil	Nil	100
		0·25	Nil	Nil	100
29. ix. 43	0·3	0·1	7·9	0·19	35
		0·15	5·9	0·12	61
		0·20	0·7	Nil	100
		0·25	Nil	Nil	100
		0·30	Nil	Nil	100
		0·40	Nil	Nil	100
8. x. 43	0·5	0·1	8·0	0·18	62·9
		0·15	6·7	0·12	76·0
		0·20	1·4	0·01	98
		0·25	Nil	Nil	100
		0·30	Nil	Nil	100
		0·40	Nil	Nil	100
19. xi. 43	0·6	0·1	7·1	0·28	53·0
		0·2	Nil	Nil	100
		0·3	Nil	Nil	100
		0·4	Nil	Nil	100
19. xi. 43	0·7	0·1	7·2	0·29	58
		0·2	Nil	Nil	100
		0·3	Nil	Nil	100
		0·4	Nil	Nil	100
11. ii. 44	0·8	0·10	7·8	0·47	40
		0·15	7·1	0·37	53
		0·20	1·6	0·02	97
		0·30	Nil	Nil	100
		0·40	Nil	Nil	100
11. ii. 44	0·9	0·10	8·05	0·5	44
		0·15	7·4	0·4	55
		0·20	0·9	Nil	100
		0·30	Nil	Nil	100
		0·40	Nil	Nil	100
11. ii. 44	1·0	0·10	8·1	0·5	50
		0·15	7·2	0·48	52
		0·20	Nil	Nil	100
		0·30	Nil	Nil	100
		0·40	Nil	Nil	100

* Mean of duplicate assays.
† A value within the expected experimental error.

A sample of this alkalinized, photolyzed blood was assayed direct at levels ranging from 0·1 ml. by increments of 0·1 to 0·5 ml. Since no growth was observed at any level it may be assumed that the treatment destroyed the riboflavin and that no other stimulatory substances are present in blood. The resulting liquid was dark brown in colour and graded quantities were added to tubes containing 0·1–1·0 μg. of riboflavin/10 ml. of medium. The work was done on three different batches of pooled blood and the results calculated as percentage loss of riboflavin (Table 5).

It appears that there is some strongly inhibitory substance added with the alkalinized photolyzed blood which can be detected with amounts of blood of 0·2 ml. or more. This 'inhibitory' effect is frequently noted in the assay of individual blood specimens (cf. Table 2). Such inhibitory substances are clearly important in the technical assay and in the interpretation of clinical riboflavin-deficiency conditions. Experiments showed that ultra-violet irradiation of the inorganic constituents added with the blood did not produce an inhibitory effect; the indication was that the inhibitory material was derived from the blood.

Effect of calcium salts. Calcium, in concentrations of 0·01–0·04 mg. and of 8–80 mg./10 ml. of medium, had no inhibitory effect.

SUMMARY

1. Some observations on the factors governing the technique of microbiological assays in general have been recorded.

2. A modified method for determining the blood riboflavin in 1 ml. of blood is described.

3. The presence of a factor or factors in blood which interferes with the microbiological assay of riboflavin has been shown.

4. The riboflavin contents of eight normal and of fifty-six rheumatic blood samples have been determined; no striking variations were found.

5. An attempt has been made to correlate the blood riboflavin with the sedimentation rate of rheumatic blood but no relationship has been established.

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Rennet Hysteresis and the Calcium Phosphate of Milk

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Heated milk exhibits a phenomenon known as rennet hysteresis, i.e. its time of coagulation shows a progressive increase with increase in the interval between heating and addition of rennet. Mattick & Hallett (1929) observed the effect in milk heated to temperatures ranging from 40 to 100°, and showed that a maximal and constant renneting time was attained in about 5 hr. Powell & Palmer (1935) later showed that the phenomenon was in all probability a property of the calcium caseinate-calcium phosphate complex of milk, demonstrable in artificial complexes of this type but not in calcium caseinate alone. They further observed that rennet hysteresis occurred both in solutions of the calcium caseinate-calcium phosphate complex to which gelatin had been added, and in gelatin-protected solutions of calcium phosphate to which calcium caseinate had been added. The nature of the protecting protein thus appeared to be a matter of indifference for the phenomenon, and the two forms of phosphate protection, by caseinate and by gelatin, seemed to differ little if at all. Earlier experiments by the writer (Pyne, 1934) had shown, on the other hand, that these two systems reacted differently with formaldehyde, whence it appeared that the nature of the protection was essentially different in the two cases; protection of calcium phosphate by caseinate in milk being, it was suggested, chemical rather than physical. In view of the apparently divergent behaviour of the system the experiments of Powell & Palmer were repeated by the author, who was unable to confirm them, the type of hysteresis being found to depend on the nature of the protecting protein. An explanation of the apparent origin of these differences is given later.

In view of these findings it was decided to examine further the whole phenomenon. The great sensitivity of the rennet coagulation to changes in the concentration of calcium ions suggested that the shift in equilibrium of the caseinate-phosphate complex postulated by Powell & Palmer might take the form of a temporary change in the concentration of these ions in milk serum as a result of heating. Heat treatment, especially if severe, is known to produce permanent changes of this kind and it seemed possible that it might also give rise to short-time changes hitherto overlooked owing to the relatively long interval generally elapsing between heating and analysis. The comparative slowness with which phosphate equilibrium is attained in milk, as exemplified by the changes in soluble calcium content which occur when milk is shaken for prolonged periods (Lampitt, Bushill & Filmer, 1937), lends support to such a suggestion.

EXPERIMENTAL

Freshly separated skim milk was used and only one heat treatment was in general employed, 80–85° for 30 min. Calcium caseinate-phosphate complexes were prepared as by Powell & Palmer (1935), colloidal calcium phosphate protected by gelatin by the method of De Toni (1921). Renneting was carried out in a thermostat at temperatures ranging from 30 to 40°, and in general without addition of calcium salts, though such addition was necessary for the artificial caseinate-phosphate-gelatin complexes. Rennet, either as the commercial liquid extract or from tablets, was used in such amounts as to give convenient renneting times (2–10 min.). Coagulation times were determined for larger samples as by Powell & Palmer (1935), and for small samples by direct observation of curd formation.

For conductivity measurements the Kohlrausch bridge and telephone with valve amplification was employed, for freezing-point determinations, the Hortvet cryoscope.

Analytical methods for calcium and phosphate, where not otherwise stated, were those used by Pyne (1940).

RESULTS

Serum changes accompanying hysteresis

The relatively short life of the hysteresis phenomenon—about 5 hr.—made it necessary to employ rapid methods of investigation in order to detect any accompanying serum changes. Physical means, especially conductivity measurements, had obvious advantages here.

(*a*) *Conductivity and other physical methods.* Preliminary experiments with heated milk were negative, the conductivity of the rapidly cooled samples remaining apparently unchanged on further standing. When, however, chlorides, which account for the greater part of the conductivity, were largely removed by dialysis, either against successive changes of artificial chloride-free milk serum, or better still, against dilute M/1400-calcium phosphate solutions of pH 6·7, a marked rise in conductivity was found to accompany the changes of renneting time throughout the hysteresis period. Milk dialyzed in this way showed, incidentally, the characteristic rennet hysteresis of normal milks. Table 1 gives some typical results.

The concentration changes suggested by conductivity measurements were too slight for detection by freezing-point determinations on milk whether dialyzed or undialyzed. An attempt to employ the polarographic method for the same purpose was also unsuccessful. With barium caseinate-barium phosphate solutions (calcium cannot be satisfactorily estimated) it was found that the colloid constituents contributed so greatly to the diffusion current as to mask any small change in barium ion concentration which might accompany hysteresis. This matter is receiving further attention.

The changes in concentration of calcium and phosphate, suggested by conductivity measurements, were next investigated more quantitatively by chemical analysis.

(*b*) *Rapid dialysis.* The following conditions were adopted. NaCl (0·25 ml. of a 1% solution) containing a trace of saponin (to ensure wetting of membrane) was placed in a dialyzer consisting of a short length of 22 mm. glass tubing sealed at one end with a stretched cellophan membrane. The dialyzer was placed in about 50 ml. of the milk under investigation and the whole gently rocked for 30 min. This time of contact ensured a diffusion of ions (50–70%) adequate for comparative purposes without allowing the dialysis time to extend too widely over the hysteresis period.

Inorganic phosphate and calcium were determined on 0·1 ml. portions of the dialysate, the former by the Kuttner & Lichtenstein technique (1930), the latter by a micro adaptation of the method of de Loureiro & Janz (1944).

The results (Table 2) show that there is a definite increase in the concentration of soluble calcium and phosphate throughout the hysteresis period.

(*c*) *Permutite action.* Davies (1937) has shown that contact of milk with barium permutite brings

Table 1. *Conductivities and renneting times at various intervals after heating of milk dialyzed against* M/1400*-calcium phosphate and heated for* 30 *min. at* 85°

	Specific conductivity (mhos × 10^{-6} at 15°) after (min.)							Renneting time at 35° (sec.) after (min.)	
	5	15	30	60	120	180	240	5	240
A	408	413	419	427	432	436	439	370	488
B	399	404	409	—	425	430	—	382	450
C	481	485	490	498	—	510	514	282	515

This definite rise in conductivity of dialyzed milk which occurs on standing after heating provided the first clear evidence that the hysteresis phenomenon was associated with a progressive increase in the amount of soluble material, the latter consisting undoubtedly of calcium and phosphate released into solution from the caseinate-phosphate complex. Incidentally the rate of change of conductivity with time, at first rapid, later slower, shows a remarkable parallelism to that of the renneting time over the hysteresis period (cf. Mattick & Hallett, 1929).

Table 2. *Calcium and phosphate contents of dialysates from heated* (85°, 30 *min.*) *milk at various intervals after heating*

		Sample						
		A	B	C	D	E	F	G
Ca (mg./100 ml.)	(5 min. after heating)	15·2	13·0	16·0	15·2	—	—	—
	(240 min. after heating)	19·6	17·2	19·6	17·2	—	—	—
Phosphate (mg. P/100 ml.)	(5 min. after heating)	26·5	25·3	—	—	33·5	23·4	24·7
	(240 min. after heating)	28·9	27·4	—	—	35·9	25·9	26·1

about, without appreciable change in reaction, a rapid replacement by barium of the soluble calcium, followed by a slower replacement of the insoluble or colloidal calcium. The method seemed applicable to the detection of the changes in the concentration of serum calcium which might accompany rennet hysteresis.

The heated milk was rapidly cooled, immediately shaken for 10 min. with one-fourth its weight of barium permutite, drawn off, centrifuged free from suspended matter, and analyzed for calcium and phosphate. A second sample of the milk was similarly treated some 4 hr. after heating and cooling.

Table 3. *Calcium and phosphate contents and* pH *values of heated* (85°, 30 *min.*) *milk treated with barium permutite at various intervals after heating.*

	Sample			
	A	B	C	D
Ca (mg./100 ml.)				
(5 min. after heating)	106	132	89·2	94·4
(240 min. after heating)	94	121	86·0	88·4
Phosphate (mg. P/100 ml.)				
(5 min. after heating)	47·5	56·8	40·2	—
(240 min. after heating)	40·5	52·5	36·6	—
pH				
(5 min. after heating)	6·49	6·76	7·00	6·53
(240 min. after heating)	6·52	6·87	7·03	6·70

In all cases the residual calcium and phosphate of the permutite-treated milks are seen to have diminished with increase in the time since heating (Table 3), i.e. the amounts of these ions taken up by the permutite has increased during the period. Freshly heated milk is thus lower in rapidly replaceable or soluble calcium and also in soluble phosphate than milk that has stood for some hours after heating. The permutite results thus confirm, at least qualitatively, those obtained by dialysis. Quantitatively the agreement between them is naturally less marked, and the values found for calcium replaced by barium are in general low compared with those reported by Davies, who, however, remarks on the very different replacement activities of different samples of permutite.

The reduction in the concentration of soluble phosphate following contact with barium permutite, observed in these experiments, suggests that the reaction between this substance and milk is not limited merely to basic exchange.

(*d*) *Rennet whey.* Analysis of rennet wheys formed at different stages of the hysteresis also indicated that the phenomenon is accompanied by an increase in soluble calcium and phosphate. In preparing these wheys, renneting was carried out at 30°, with addition of 1% of commercial liquid extract. A curd sufficiently firm to yield a clear whey by centrifuging and filtration was obtained in 20–30 min.

Analyses of rennet curd (Grimmer & Paape, 1930) and of whey (Pyne, 1940) suggest that an adsorption of soluble calcium and phosphate occurs at some time during the renneting process. The analyses recorded (Table 4) are therefore presumably composite values resulting from the superimposition of this tendency on the contrary changes properly due to hysteresis. Nevertheless, the characteristic feature of the hysteresis phenomenon—the progressive release into solution of calcium and phosphate—is again evident.

Composition of the adsorbed and subsequently released phosphate

The amounts of calcium and phosphate released from the caseinate-phosphate complex during the hysteresis period are small. Nevertheless, it is possible from consideration of the data in Tables 2 and 4 to form a rough estimate of the type of phosphate involved in these changes. The data, where complete, suggest a Ca/P ratio of approximately 2 (Ca/P for $Ca_3P_2O_8 = 1{\cdot}94$); the phosphate released therefore appears to correspond to that normally present in the calcium caseinate-calcium phosphate complex (Pyne, 1934). Indirect evidence that release of a relatively alkaline phosphate actually occurs during hysteresis is furnished by the pH values of the various wheys and permutite filtrates (Tables 2 and 4), since the course of the hysteresis is seen to be accompanied by a slight shift to a more alkaline reaction.

Table 4. *Calcium and phosphate contents and* pH *values of rennet whey prepared at* 30° *from milks at various intervals after heating* (85°, 30 *min.*)

		Sample				
		A	B	C	D	E
Ca (mg./100 ml.)	(5 min. after heating)	37·8	36·4	—	—	—
	(240 min. after heating)	40·6	38·8	—	—	—
Phosphate (mg. P/100 ml.)	(5 min. after heating)	39·8	38·5	27·5	37·6	31·5
	(240 min. after heating)	41·0	39·8	28·6	39·3	33·1
pH	(5 min. after heating)	6·38	6·52	—	—	—
	(240 min. after heating)	6·51	6·61	—	—	—

The relation of hysteresis to the various stages of rennet action

Rennet reaction is generally regarded as occurring in two stages: (*a*) enzymic scission of the caseinate (or alternatively digestion of some protecting substance) with formation of paracasein, (*b*) coagulation of paracasein by calcium ions. Some adsorption of calcium is apparently involved in the second stage, judging from the composition of rennet curd (Grimmer & Paape, 1930) and whey (Pyne, 1940). Rapid coagulation is in general associated with higher concentrations of ionic calcium and vice versa.

It has been seen that rennet hysteresis is, on the other hand, characterized by a progressive decrease in the ability of milk to be coagulated by rennet, accompanied by a corresponding increase in soluble calcium content. This somewhat unexpected result suggests that it is not the concentration of soluble calcium *per se* which plays the essential part in the second stage of the rennet reaction but rather the amount of calcium adsorbed (presumably as phosphate) from solution. Moreover, calcium adsorbed in these hysteresis experiments as a result of the action of heat, and thus prior to the addition of enzyme, appears to be as effective in promoting coagulation as is calcium, adsorbed after the first stage of the rennet reaction, on the ordinary view of the renneting process.

In order to ascertain more definitely to what extent either or both stages of the rennet reaction were influenced by this previously adsorbed calcium phosphate a series of renneting experiments was carried out. Heated milk, immediately after cooling, was treated with rennet (1·25 % of a 2·5 % solution prepared from Benger tablets) at about 16°, a temperature low enough to allow the first part of the reaction, but not, to any appreciable degree, the second, since this has a much higher temperature coefficient (Berridge, 1942). After being held at 16° for various periods, small samples were transferred to copper vessels and rapidly heated by immersion and gentle rocking in a water-bath at 40° in order to bring about the second or so-called calcium ion precipitation stage. Time of coagulation was taken as that required for the first appearance of particles of curd.

A second sample of the same milk which had been allowed to stand for some hours at ordinary temperatures, after heating and cooling, was similarly treated. Table 5 gives the average relative coagulation times at 40° for a series of six milk samples submitted to this treatment. The initial coagulation time of the sample renneted immediately after heating and cooling is taken as 100 in all cases.

Table 5. *Average relative coagulation times at* 40° *of heated and rapidly cooled milk which had, at two very different intervals after heating* (85°, 30 *min.*) *been submitted to rennet action at* 16° *for various times*

Duration of rennet treatment at 16°		Min. ½	5	10	15	20	25	30	40	50	60
Time of subsequent coagulation at 40° of milks treated with rennet	(5 min. after heating)	100·0	70·8	58·6	49·7	48·7	48·2	49·2	49·2	50·0	51·8
	(240 min. after heating)	157·4	125·5	112·0	95·7	91·2	87·0	83·5	82·2	77·3	73·4

These results suggest that rennet hysteresis has its origin in the second or calcium precipitation stage, for all the milk samples show a rapid and fairly uniform fall in renneting time to a moderately constant value in about the same time—some 15 min. in these experiments—corresponding undoubtedly to the completion of the first or enzymic stage. Thereafter, the course of the renneting times diverges somewhat for the two groups. The milk submitted to rennet action 4 hr. after heating shows a further but much more gradual fall in renneting time; milk which has been renneted immediately after heating maintains, on the contrary, a renneting time which is almost unchanged but shows a slight tendency to rise again. This behaviour on the part of freshly heated milk is undoubtedly due to the supervention of the hysteresis phenomenon. The longer periods of contact with rennet at 16°, by permitting a greater degree of re-solution of adsorbed calcium phosphate, can thus give rise to a slower coagulation when the sample is later warmed to a temperature at which the second stage of the reaction can occur. It is of interest to note that this loss of calcium phosphate into solution, leading to a retardation of the coagulation, can proceed simultaneously with and apparently independently of the first or enzymic stage of the rennet reaction.

That the hysteresis phenomenon is associated with the second stage of the rennet reaction is further attested by the following facts: (*a*) milk heated to temperatures as low as 40–43° shows some degree of hysteresis, without, however, suffering any permanent reduction in renneting ability as compared with the corresponding raw milk (Mattick & Hallett, 1929). Hysteresis can thus occur under circumstances where no injury to the enzymic reaction appears to have taken place; (*b*) again, differences in renneting time are observed also in milk subjected to different heat treatments even where these are so mild (holding at 0–2° and

at 30° respectively) as to exclude the possibility of injury to the enzymic reaction proper (see Table 6). The samples investigated here were first of all submitted to flash pasteurization at 75° in order to obviate possible bacterial change during the subsequent holding at the lower temperatures; (*c*) the extreme sensitiveness of the renneting time of artificial calcium caseinate-calcium phosphate complexes to the previous heat treatment of these complexes, even where this is relatively mild (Holter, 1932), is apparently an instance of the same kind, accentuated probably by the dilute nature of the solutions investigated.

Table 6. *Coagulation times with rennet at* 40° *of milk previously held at different relatively low temperatures*

	Coagulation times (sec.)		
	A	B	C
Held 24 hr. at 0–2°	106	162	452
Held 24 hr. at 0–2°, then 5 hr. at 30°	88	122	306

Hysteresis in calcium caseinate-calcium phosphate-gelatin systems

As mentioned earlier, the present investigation owes its inception to divergent observations on the behaviour of these heated systems to rennet. The results of Powell & Palmer (1935) with mixtures of calcium caseinate and gelatin-protected calcium phosphate indicate the existence of a normal hysteresis similar to that of milk; those of the writer, on the other hand, indicate a reversed hysteresis (Table 7).

Table 7. *Average coagulation times with rennet at various intervals after heating* (85°, 30 *min.*) *of different types of calcium caseinate-phosphate-gelatin systems*

System	No. of exp.	Average coagulation time at 35° (sec.) after (min.) 5	240
Calcium caseinate-calcium phosphate complex + gelatin	9	104	133
Calcium phosphate protected by gelatin + calcium caseinate	11	184	157

The mixtures investigated contained in general 2·5% calcium caseinate, 2·5% gelatin, and were about M/300–M/150 in $Ca_3P_2O_8$. Owing to their low content of soluble calcium it was necessary, following Powell & Palmer, to add a small quantity of a soluble calcium salt, 2–5% of 0·14M-$CaCl_2$, just before renneting in order to obtain satisfactory coagulation. Sufficient rennet extract was then added to cause coagulation in about 2–5 min. at 35°.

The divergent observations on the hysteresis of these systems appear to have their origin in differences in the methods of preparing the gelatin-protected calcium phosphate. The writer, following De Toni (1921), prepared the colloid by slowly running a solution of trisodium phosphate into a boiling gelatin solution of calcium chloride. De Toni emphasizes the need for boiling, without which combination between calcium and phosphate proceeds too slowly in the presence of gelatin to form appreciable amounts of the inorganic colloid. Prepared in this way the colloid forms an almost opaque, porcelain-like solution. Powell & Palmer's preparation, on the other hand, appears to have been made in the cold from lime and phosphoric acid solutions. It is said to have been translucent—a description which implies that its formation was at least incomplete. Its behaviour on heating is in accordance with this supposition. A marked shift in reaction towards acidity was found to occur, suggesting that the formation of tricalcium phosphate, accompanied by that of the monocalcium salt, was at this stage taking place from the elements of the dicalcium salt. If this view is correct, it would appear probable that when this gelatin-calcium phosphate solution was added to one of calcium caseinate and the mixture heated for the study of its hysteresis, some formation of tricalcium phosphate also took place, but this time in the presence of calcium caseinate. Consequently the system obtained in this way is unlikely to have differed essentially from that prepared by adding gelatin to the calcium caseinate-calcium phosphate complex. That both should have shown the same type of hysteresis is therefore not surprising.

From the present experiments it is clear that the hysteresis phenomenon is not identical for caseinate-gelatin-phosphate systems in which the phosphate is protected by caseinate and by gelatin respectively. The equilibrium between dissolved calcium phosphate and the complex appears to differ, as regards the direction and magnitude of its displacement by heat treatment in the two cases. The nature of the protection exercised by the two proteins can therefore hardly be the same.

DISCUSSION

It is clear from the experiments here reported that rennet hysteresis merely reflects a general phenomenon of milk—a heat-induced displacement of the equilibrium between calcium phosphate in solution and the colloidal calcium caseinate-calcium phosphate complex. Rise of temperature produces a temporary transfer of some of the soluble calcium and phosphate to the colloid complex, and this transfer is slowly and (if the heating has been more than moderate) only partly reversed on subsequent

cooling; chilling produces naturally the opposite effect (see Table 6). Other things being equal, the greater the degree of adsorption of calcium phosphate by the complex, the more rapidly will the system coagulate when acted upon by rennet. The enzymic attack proper is not, however, concerned in these changes, which appear instead to influence the renneting time mainly, if not exclusively, by their effect on the second or so-called calcium ion precipitation stage. Severe heat treatment, of course, can independently affect the sensitivity of the caseinate molecule to rennet enzyme attack and retard the coagulation as a whole. This, however, is apparently an additional effect of heating and does not in general mask the hysteresis phenomenon unless it is so severe as to inhibit coagulation altogether.

It is difficult to reconcile these results with the view that the enzymic part of rennet action consists of an alteration of a constituent of the original casein which acts, before this alteration, as a protective colloid for the main cation-sensitive group of constituents (Linderstrøm-Lang, 1928; Holter, 1932). Adsorption of calcium phosphate from solution by the caseinate complex as a result of heat treatment seems to affect the second stage (while leaving the first stage of the reaction unaffected) in much the same way as if the adsorption had occurred during this stage and subsequent to the enzyme reaction proper. This behaviour appears to conform with the older Hammarsten view of rennet action.

As to the differences observed in the type of hysteresis exhibited by caseinate-phosphate-gelatin systems according to whether caseinate or gelatin is the protecting colloid, the facts are best explained by assuming that the caseinate-phosphate complex is essentially a calcium caseinate-phosphate, i.e. a compound in which the union of phosphate and caseinate has taken place through calcium atoms common to both. The gelatin-protected phosphate would, on the other hand, appear to resemble a gold sol, and to consist presumably of a calcium phosphate nucleus coated with gelatin. Obviously the composition of the postulated caseinate-phosphate will not be fixed, but can vary according to the number of phosphate groups present. Moreover, the heterogeneity of casein is not relevant in either case; there is no suggestion that all the casein fractions are equally capable of forming these complexes.

SUMMARY

1. The rennet hysteresis of heated milk arises from the adsorption of calcium phosphate by the calcium caseinate-calcium phosphate complex during heating, followed by its gradual release again at lower temperatures.

2. Calcium phosphate thus adsorbed affects the renneting time primarily through its acceleration of the second or so-called calcium ion precipitation stage of the coagulation. This result seems to accord best with the Hammarsten theory of rennet action.

3. Rennet hysteresis of calcium caseinate-calcium phosphate-gelatin systems differs according to whether the phosphate is protected by caseinate or by gelatin. This behaviour is interpreted as favouring the existence of a chemical union between the two constituents in the calcium caseinate-calcium phosphate complex of milk.

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The Distribution of Myrobalanitannin

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Myrobalanitannin (I: luteoic acid 5-biglucoside, $R = C_{12}H_{21}O_{10}$) present in myrobalans, the fruit of *Terminalia chebula* Retz., was first obtained by Nierenstein (1910) as a well-crystallizing substance which gave, on hydrolysis either with dilute sulphuric acid or with emulsin, 1 mol. ellagic acid (II) and 2 mol. glucose. The hydrolysis with emulsin excludes the possibility that myrobalanitannin is

an acyl derivative (Fischer & Bergmann, 1918) but not the possibility that it is a diglucoside (e.g. III: luteoic acid 4:5-diglucoside, $R = R_1 = C_6H_{10}O_5$), and we have therefore methylated myrobalanitannin with diazomethane and hydrolyzed the product with dilute sulphuric acid. We obtained tetramethylellagic acid (IV), which thus confirms the original structure (I).

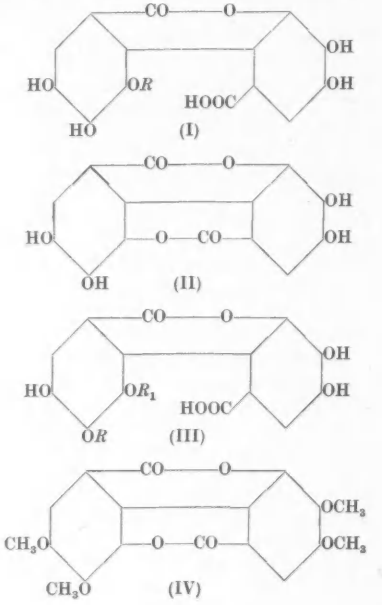
Table 1. *Isolation of myrobalanitannin from various sources*

	Yield (%)	Country of origin	Official name	Local name	Analysis* Glucose (%)	Analysis* Ellagic acid (%)
(i) Roots, rhizomes or underground stems:						
Ailanthus excelsa Rxb.	2·4	India	—	—	49·6	48·3
Donabanga mollucana Blr.	1·2	Java	—	—	49·5	48·4
Geranium maculatum L.	1·8	U.S.A.	—	—	49·7	48·6
G. wallachium Don.	2·0	France	—	—	49·3	48·7
Nupher luteum Sib. & Sm.	2·2	France	—	—	49·5	48·6
Nymphaea alba L.	0·5	Asia Minor	—	—	49·3	48·6
N. adorata Ait.	1·4	U.S.A.	—	—	49·4	48·6
Statica brasiliensis Bois	0·9	Chile	*Radix Bayeura*	—	49·2	48·3
St. caroliniana Willd.	1·1	Venezuela	—	—	49·3	48·2
St. Gmelinii Willd.	3·0	Caucasus	—	—	49·5	48·7
(ii) Barks:						
Ailanthus glandulosa Desf.	0·9	Japan	—	—	49·3	48·6
Aralia spinosa L.	1·81	U.S.A.	—	—	49·5	48·5
Aulomyrica ramulosa Bag.	1·1	Brazil	—	—	49·3	48·6
Companesia guaviroba† B. & H.	2·2	Paraguay	—	—	49·7	48·6
C. aphyla James & Nies.	1·71	U.S.A.	—	—	49·6	48·3
Daniella thurifera Beum.	1·98	Gold Coast	—	—	49·3	48·7
Eugenia opiculata DC.	3·2	Venezuela	—	—	49·5	48·2
E. jambolana Lam.	1·2	Mauritius	*Cortex Sygii Jambolanii*	—	49·2	48·5
Punica granatum L.	3·2	Egypt	*Cortex granatum*	—	49·4	48·7
Somodera indica Gaertn.	1·9	Ceylon	—	—	49·5	48·3
Sacrocephalus esculentus Afz.	5·4	Togoland	*Lignum Njimo*	Doundaka	49·3	48·5
Terminalia chebula Retz.	6·3	India	*Cortex Myrobalanii*	—	49·5	48·7
T. angustifolia Douglas & Nies.	2·5	Northern Rhodesia	—	—	49·4	48·4
Woodfordia floribunda Salisb.	11·8	India	—	—	49·5	48·3
W. siderafolia Nies.	1·5	Uganda	—	—	49·6	48·7
Rhizophera Ikotae Nies.	2·9	Northern Rhodesia	—	Majunji	49·3	48·5
(iii) Leaves:						
Acrostaphyllos uva-ursi Spr.	1·1	Northern Russia	*Foliae uvae ursi*	—	49·6	48·3
A. glauca Lindl.	3·2	California	—	Manizato	49·3	48·4
A. alba Nies.	3·3	U.S.A.	—	—	49·4	48·5
A. arjuma mississippiana Nies.	4·3	U.S.A	—	—	49·5	48·3
Datura arborea L.	1·2	Peru	—	—	49·6	48·5
Ourouparia rhynchophylea Mats.	0·5	Japan	—	—	49·5	48·3
(iv) Fruits:						
Caesalpinia coriaria Willd.	14·3	Mexico	—	Divi-Divi	49·6	48·5
C. brevifolia Boil.	16·2	Chile	*Balsamo carpon*	Algarobilla	49·2	48·7
C. melanocarpon var. *ugandae* Nies.	13·3	Uganda	—	Mbalimbali	49·4	48·6
Haematoxylon campechianum L.	1·2	Jamaica	—	—	49·6	48·3
Terminalia bellarica Roxb.	12·4	India	*Myrobalani bellaricae*	—	49·5	48·4
T. citrina Roxb.	28·2	India	*Myrobalani citrinae*	—	49·3	48·2
T. chebula Retz.	21·2	Nyasaland	—	Cabazoon	49·6	48·7
(v) Galls						
Terminalia chebula Retz.	11·3	India	—	Dokojo nuts	49·4	48·6
Potentilla reptans L.	0·9	England	—	Hedge bank gall	49·7	48·5
Ajuga reptans L.	0·3	England	—	Pitmaking gall	49·4	48·7
Quercus sessiliflora Sal.	1·4	England	—	Two-cell gall	49·5	48·5
(vi) Buds						
Eugenia caryophyllata Thunb.	2·2	Madagascar	*Flores caryophyllii*	—	49·5	48·6
Liriosima orata Miers.	1·1	Brazil	—	Muriapuama	49·3	48·3
Apidosperma sessiliflora Allem.	3·2	Chile	—	—	49·2	48·5
(vii) Cupulae:						
Quercus aegilops L.	28·2	Greece	—	Valonea	49·3	48·3
Q. prinus L.	17·5	U.S.A.	—	—	49·7	48·5
Quercus sp.	28·2	Uganda	—	—	49·4	48·7

* Calculated values for myrobalanitannin $C_{76}H_{52}O_{46}$: glucose, 49·4; ellagic acid, 48·5%. For further identification the samples were methylated with diazomethane and hydrolyzed; in each case tetramethyl ellagic acid was obtained, m.p. and mixed m.p. 289–290°.

† Frequently incorrectly referred to as *Companosia guavira*.

Since the original isolation of myrobalanitannin in 1910 this tannin has also been identified in witch-hazel bark (Edwards & Nierenstein, 1943), and, as shown in this communication, in a large number of other plants. As will be seen (Table 1), the bark of *T. chebula* also contains myrobalanitannin, which thus disproves the contention of Meyer (1909) that the tannin yielding ellagic acid is not present in the bark.



EXPERIMENTAL

I. *Confirmation of formula (I).* Myrobalanitannin was methylated with excess diazomethane in an apparatus similar to that described by Malkin & Nierenstein (1930) and the methanol solution refluxed for 6–8 hr. on a boiling water-bath with a solution of sulphuric acid in dilute methanol. Recrystallization of the product from methanol gave tetramethylellagic acid in microscopic needles, m.p. 289–290°. (Found: C, 60·6; H, 4·1; OCH_3, 34·2%. Calc. for $C_{18}H_{14}O_8$: C, 60·4; H, 4·0; OCH_3, 34·1%.)

II. *Distribution of myrobalanitannin.* The finely powdered materials were extracted with a mixture of chloroform and carbontetrachloride, so as to remove fats, waxes, etc., and then percolated with ethanol until no more tannin could be qualitatively detected. The residue left on evaporation of the alcohol crystallized from distilled water in faintly brownish microscopic needles, which did not melt below 360°. For myrobalanitannin Edwards & Nierenstein (1943) record $[\alpha]_D^{21°} = +21·98°$ (water); we found $[\alpha]_D^{21°} = +21·77°$ (water), $[\alpha]_D^{23°} = +37·16°$ (ethanol) and $[\alpha]_D^{17°} = +29·03°$ (methanol). The distribution of myrobalanitannin is given in Table 1. Glucose and ellagic acid were estimated by the method of Nierenstein, Spiers & Geake (1921).

SUMMARY

The original formula of myrobalanitannin has been confirmed, and the distribution of this tannin studied.

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Interfering Substances in the Roe and Kuether Method for the Determination of Ascorbic Acid

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Roe & Kuether's method (1943) is based upon the red coloration given by the 2:4-dinitrophenylhydrazine derivative of dehydroascorbic acid with 85% H_2SO_4. This reaction is very sensitive and its specificity, as will be shown, is high. We investigated the method in connexion with another inquiry and record some of our observations.

METHODS

General technique

Our technique was that of Roe & Kuether (1943), but we allowed the final coloured solution to stand for ½ hr. at constant temperature, in order to minimize the possibility of error due to fading when investigating interfering substances. All estimations were carried out in 4% trichloroacetic acid and the colour developed was measured in an Evelyn type photoelectric colorimeter. The standard error of percentage deviation from the mean of individual *l*-ascorbic acid determinations over a period of 6 months for the range 0·001–0·04 mg. *l*-ascorbic acid/4 ml. was 2%. The use of different batches of reagents did not influence the results. A linear relationship between log R and concentration was found up to about 0·01 mg. *l*-ascorbic acid/4 ml., in reasonable agreement with the above authors. When the Evelyn type of photoelectric colorimeter was replaced by the 'Spekker' Absorptiometer (standard or sensitive type), or when King & Haslewood's (1937) visual photometric technique was employed, a lower limit of only 0·01 mg./4 ml. could be satisfactorily attained.

Reagents

l-*Ascorbic acid*, *reductic acid* and d-*glucoascorbic acid* (see Snow & Zilva, 1941).

Reductone (see Snow & Zilva, 1938).

d-*Araboascorbic acid* (see Snow & Zilva, 1943).

2:3-*Diketogulonic acid solution* (see Penney & Zilva, 1943).

Laboratory reagent quality 2:4-dinitrophenylhydrazine, thiourea, and trichloroacetic acid were used. The sodium carbonate, bicarbonate and hydroxide, and the glucose, were Analar reagents. Analar sulphuric acid was used to prepare an 85% solution (Roe & Kuether, 1943). The norite was a commercial sample of activated vegetable charcoal washed with acid before use.

Heat treatment of alkaline solutions of glucose

Alkaline solutions of glucose were prepared using: (1) $NaHCO_3$, (2) Na_2CO_3, (3) 0·3 N-NaOH, (4) 0·02 N-NaOH.

(1) $NaHCO_3$: (*a*) a 1% solution of glucose in 0·5% $NaHCO_3$ was boiled for 30 min. (see Roe & Oesterling, 1944); (*b*) equal volumes of 2% glucose and 1% $NaHCO_3$ solutions were heated in a boiling water-bath until a constant temperature was attained. After mixing, the heat treatment was continued for 30 min.; (*c*) equal volumes of 2% glucose and 1% $NaHCO_3$ solutions, after being heated to 90°, were mixed and allowed to react at that temperature for 30 min. After heating, the solutions were made slightly acid with HCl, cooled and diluted to the required volume.

(2) Na_2CO_3: glucose solutions in Na_2CO_3 were treated as above.

(3) 0·3 N-NaOH (see Snow & Zilva, 1944): equal volumes of 4% glucose solution and 0·6 N-NaOH were heated to 90° in a water-bath and then mixed. After ½ min. at this temperature, the mixed solutions were removed, acidified, cooled and diluted to the required volume.

(4) 0·02 N-NaOH (see Snow & Zilva, 1944): equal volumes of 4% glucose solutions and 0·04 N-NaOH were treated as above except that the mixed solutions were allowed to remain at 90° in the water-bath for 10 min.

All the above alkaline solutions were coloured yellow-brown after the heat treatment, the intensity of the coloration varying directly with the severity of the treatment. On acidification some, but not all, of the colour was dispersed.

Direct titrations with indophenol were carried out at pH 1·5.

RESULTS

Interfering substances

Reductic acid. When the Roe & Kuether reaction is applied to reductic acid, a colour develops which is similar to that obtained with *l*-ascorbic acid. There is, however, one essential difference, namely, that if the reaction is carried out at 25° for 15 min. about 92% of the colour given after heating at 37° for 3 hr. is generated. This can be seen from Table 1.

Table 1. *The production of colour by reductic acid after 15 min. at 25° and 3 hr. at 37°*

Exp.	Reductic acid (mg.)	'Ascorbic acid' found at 25° (mg.)	'Ascorbic acid' found at 37° (mg.)	'Ascorbic acid' at 25° / 'Ascorbic acid' at 37° (%)
1	0·008	0·0084	0·0093	90
2	0·008	0·0089	0·0096	93
3	0·008	0·0091	0·0096	95
Average	0·008	0·0088	0·0095	92

Table 2. *Recovery of l-ascorbic acid in the presence of reductic acid*

l-Ascorbic acid added (mg.)	Reductic acid added (mg.)	'Ascorbic acid' found at 25° (mg.)	'Ascorbic acid' found at 37° (mg.)	*l*-Ascorbic acid after correction (mg.)	Error before correction (%)	Error after correction (%)
0·020	0·008	0·0087	0·0288	0·0201	+44	+ ½
0·020	0·016	0·0174	0·0383	0·0209	+91	+4½

Ascorbic acid under the same conditions generates only about 2·5% of its final colour. It should thus be possible to estimate *l*-ascorbic acid in the presence of reductic acid if no other interfering substances are present. That this can be done within certain limits will be seen from Table 2. The figures show that in pure solutions the error due to reductic

Table 3. *The influence of increasing amounts of reductic acid on the calculated error*

l-Ascorbic acid (mg.)	Reductic acid (mg.)	*l*-Ascorbic acid after correction (mg.)	Error (%)
0·01	0·01	0·0106	+ 6
0·01	0·02	0·0114	+14
0·01	0·03	0·0122	+22
0·01	0·04	0·0130	+30

acid, if the latter is present in amounts of the same order as *l*-ascorbic acid, can be largely eliminated. When, however, the reductic acid is higher, the error increases. This is seen in Table 3 in which the approximate figures given are calculated by the summation of the individual colour values.

Purified reductone. The solubility of the 2:4-dinitrophenylhydrazine derivative of this substance in the concentration of H_2SO_4 employed by Roe & Kuether is low. By using reasonably dilute solutions of reductone, however, this difficulty can be overcome, although under these conditions the derivative does not produce its maximum colour. By carrying out the Roe & Kuether reaction with such dilute solutions for 15 min. at 25°, about 50% of the final colour developed in 3 hr. at 37° is generated (Table 4). When, however, *l*-ascorbic acid was added to solutions of reductone and the ratio of *l*-ascorbic acid to reductone was 4 : 1 or more, it was found that *l*-ascorbic acid could be determined with a maximum error of the order of 6% by deducting the value of 'ascorbic acid' obtained at 25° from the final value at 37° (Table 5). Under these conditions the colour generated by the ascorbic acid at 25° roughly compensates for the incomplete reductone reaction.

Heated alkaline glucose solutions. Roe & Oesterling (1944) have already observed that when a 1% solution of glucose containing 0·5% $NaHCO_3$ was boiled for 30 min., a value for 'ascorbic acid' was obtained by their method which was higher than the direct indophenol titration, but when the heating was continued for 90 min. both methods gave results of a similar order. In view of these observations we decided to compare the colour reaction produced after 15 min. at 25° and after 3 hr. at 37° by alkaline glucose solutions submitted to various temperatures. The results are given in Table 6. It will be seen that in most cases the values for 'ascorbic acid' obtained by the Roe & Kuether method show fair agreement with those obtained by direct indophenol titration. Although by deducting the figures given by the reaction for 15 min. at 25° the result would still be misleading, the error would be minimized. Even then the corrected figures would indicate the presence of 'ascorbic acid' to the extent of 34% in the $NaHCO_3$ and Na_2CO_3 solutions, and 55% in the NaOH solutions, of the direct indophenol titra-

Table 4. *The production of colour by purified reductone after 15 min. at 25° and 3 hr. at 37°*

Reductone (mg.)	'Ascorbic acid' found at 25° (mg.)	'Ascorbic acid' found at 37° (mg.)	'Ascorbic acid' at 25° / 'Ascorbic acid' at 37° (%)
0·005	0·0016	0·0035	46
0·005	0·0017	0·0035	49
0·0025	0·0007	0·0015	47
0·0025	0·0008	0·0016	50
		Average	48

Table 5. *Recovery of* l*-ascorbic acid in the presence of purified reductone*

l-Ascorbic acid added (mg.)	Reductone added (mg.)	'Ascorbic acid' found at 25° (mg.)	'Ascorbic acid' found at 37° (mg.)	*l*-Ascorbic acid after correction (mg.)	Error: Before correction (%)	Error: After correction (%)
0·020	0·005	0·0021	0·0234	0·0213	+17	+6½
0·010	0·0025	0·0011	0·0118	0·0107	+18	+7
0·020	0·0025	0·0012	0·0208	0·0196	+ 4	−2
0·020	0·0025	0·0014	0·0211	0·0197	+ 5½	−1½

Table 6. *The production of colour by heat-treated alkaline glucose solutions after 15 min. at 25° and 3 hr. at 37°*

Solution	Heat treatment	'Ascorbic acid' found at 25° (mg./100 ml.)	'Ascorbic acid' found at 37° (mg./100 ml.)	'Ascorbic acid' at 25° / 'Ascorbic acid' at 37° (%)	'Ascorbic acid' by direct indophenol titration (mg./100 ml.)
$NaHCO_3$ (0·5%)	At boiling-point for 30 min.	10·0	14·5	69	15·6
		11·9	18·0	66	18·4
	At 90° for 30 min.	1·08	1·55	70	1·8
	In boiling water-bath for 30 min.	3·5	5·5	63	4·4
Na_2CO_3 (0·5%)	At boiling-point for 30 min.	23·0	33·1	70	40·0
		27·0	38·2	71	44·0
	At 90° for 30 min.	12·3	19·0	65	20·0
	In boiling water-bath for 30 min.	19·0	27·5	70	28·7
0·3 N-NaOH	At 90° for ½ min.	10·8	23·5	46	25·0
		9·9	21·9	45	24·4
0·02 N-NaOH	At 90° for 10 min.	5·3	11·75	45	11·3
		7·1	15·0	47	16·4

tion and of the uncorrected Roe & Kuether values. As has been pointed out by Snow & Zilva (1944), if their correction formula is applied to the Lugg method the error arising from the presence of the products formed in the NaOH-treated glucose solutions is also not eliminated but is greatly reduced. The application of the Lugg method would, as will be shown later, in this case be more suitable than that of Roe & Kuether although the latter offers additional qualitative information. It is of interest to note that, whilst Lugg's method indicates a difference between the heat-treated alkaline solutions of glucose prepared with 0·3N- and 0·02N-NaOH (Snow & Zilva, 1944), the behaviour of each of these solutions in the colour reaction is the same. Results obtained by us from the application of the corrected Lugg method to glucose solutions treated with $NaHCO_3$ and Na_2CO_3, such as were used in the colour reaction experiments, showed that the error due to the products obtained can be reduced to 6%. Table 7 gives the recovery of *l*-ascorbic acid added to the variously treated alkaline glucose solutions. It will be seen that the colour obtained is additive and that, providing the ratio of alkaline glucose to ascorbic acid is less than one to five, the maximum error is 6%. This is in agreement with the expected error calculated by summation.

DISCUSSION

The main fact observed in these experiments is a differential rate of reaction of the primary oxidation products of *l*-ascorbic acid, reductic acid, purified reductone and the products of the heat treatment of alkaline solutions of glucose with 2:4-dinitrophenylhydrazine. After 15 min. at 25°, using the Roe & Kuether reaction, *l*-ascorbic acid, reductic acid, purified reductone, heat-treated $NaHCO_3$ and Na_2CO_3 solutions of glucose and heat-treated NaOH solutions of glucose generate 2·5, 92, 48, 66 and 45% respectively of the colour given in 3 hr. at 37°. It therefore follows that in a known mixture of any one of the above and *l*-ascorbic acid a correction can be applied for the interfering substance. In an unknown solution, however, the error due to these substances can be reduced but not eliminated, which is more or less the case with Lugg's method if the correction of Snow & Zilva (1944) is applied. In view of this it would be of interest to compare the two procedures. Lugg's method is based on the fact that *l*-ascorbic acid and other indophenol-reducing substances combine with HCHO at different rates according to the pH of the reacting solution and thus lose their capacity for reducing the indicator.

Table 7. *Recovery of* l*-ascorbic acid in the presence of the products obtained from the heat treatment of alkaline glucose solutions*

l-Ascorbic acid added (mg.)	'Ascorbic acid' obtained by direct indophenol titration from heat-treated alkaline glucose solutions: Solution	'Ascorbic acid' (mg.)	'Ascorbic acid' found: at 25° (mg.)	'Ascorbic acid' found: at 37° (mg.)	*l*-Ascorbic acid after correction (mg.)	Error: Before correction (%)	Error: After correction (%)
0·020	$NaHCO_3$	0·0024	0·0022	0·0229	0·0207	+14½	+3½
0·020	Na_2CO_3	0·0033	0·0030	0·0238	0·0208	+19	+4
0·020	0·3N-NaOH	0·0040	0·0026	0·0235	0·0209	+17½	+4½
0·020	0·02N-NaOH	0·0042	0·0031	0·0245	0·0214	+22½	+7

Analogues of l*-ascorbic acid.* *d*-Araboascorbic acid and *d*-glucoascorbic acid when submitted to the Roe & Kuether procedure for 15 min. at 25° and for 3 hr. at 37° gave colours similar to *l*-ascorbic acid, the intensity corresponding approximately to that given by equimolar amounts of ascorbic acid.

2:3-*Diketo*-l-*gulonic acid.* Solutions of this substance gave the reaction, the intensity of the colour generated at both temperatures being similar to that given by equimolar quantities of ascorbic acid.

The following common ketonic substances do not interfere with Roe & Kuether's method, since they give a yellow coloration with 2:4-dinitrophenylhydrazine on solution in 85% H_2SO_4: pyruvic acid, acetoacetic ester, acetone, cyclohexanone, salicyl aldehyde, formaldehyde, methyl hexyl ketone, vanillin and *p*-hydroxyphenyl pyruvic acid.

At pH 1·5 *l*-ascorbic acid combines only very slowly with HCHO, whilst at pH 3·5 the combination is rapid. Pure reductone, reductic acid and heat-treated alkaline solutions of glucose, however, react at a much slower rate than *l*-ascorbic acid at pH 3·5. In comparing the two methods useful information can be obtained if the following four possibilities be considered. The symbols used in the case of the Lugg method are:

v_1 = titre after reaction at pH 1·5 with HCHO,
v_2 = titre after reaction at pH 3·5 with HCHO.

In the colorimetric procedure the limit chosen for the percentage of colour generated in 15 min. at 25° is such that, in the presence of the above substances, the final error in the assessment of ascorbic acid would be within 5%.

When $v_2 < v_1/6$ and the colour reaction at 25° < 5 % of the final colour. Both the formalin and the 2:4-dinitrophenylhydrazine methods indicate the absence of interference from the above substances. Hence the figures given for *l*-ascorbic acid are in all probability reliable. It should be borne in mind, however, that neither method corrects for the analogues of *l*-ascorbic acid and that any 2:3-diketo-*l*-gulonic acid present will interfere in the colorimetric method.

When $v_2 > v_1/6$ and the colour reaction at 25° > 5 % of the final colour. Both methods in this case suggest that one or more of the substances, such as reductic acid, purified reductone and the products resulting from the heat treatment of alkaline solutions of glucose, may be present. It therefore remains to consider the limitations of each method under these conditions. Snow & Zilva (1944) have shown that, when purified reductone is present, the error due to this substance is 5 % or less if their correction is applied in the Lugg procedure. When the reaction is carried out at pH 2·0 instead of 3·5 (cf. Mapson, 1943 *a*, *b*), the error is 7 %. The interference due to the reducing substances produced by the heat treatment of NaOH solutions of glucose can also be diminished by the corrected Lugg procedure, the errors in this case being 8 and 22 % when the NaOH concentrations are 0·02 and 0·3N respectively. When, however, our differential colorimetric procedure is used, only about 50 % of the interfering substance can be eliminated. In the case of heat-treated $NaHCO_3$ and Na_2CO_3 solutions of glucose we have found that the error due to these substances can be reduced to 6 % by the corrected Lugg method whilst the Roe & Kuether method, after correction, still gives an error of 30 %. Thus it will be seen that in all the above cases, the Lugg procedure gives a more reliable estimate of the *l*-ascorbic acid present. This is not the case if reductic acid is the interfering substance, since it has been pointed out by Snow & Zilva (1944) that corrections to the Lugg procedure applied for this substance in unknown solutions are unreliable. The colorimetric method, on the other hand, reduces the error to below 6 % if the reductic acid is present in amounts equal to *l*-ascorbic acid or less.

When $v_2 > v_1/6$ and the colour reaction at 25° < 5 % of the final colour. In this case interference is indicated only by the Lugg method. No evidence is obtained concerning the identity of these interfering substances. It is, however, clear that reductic acid, purified reductone or the products obtained by the heat treatment of alkaline glucose solutions are not the cause of interference since the reaction with 2:4-dinitrophenylhydrazine for 15 min. at 25° is very small.

When $v_2 < v_1/6$ and the colour reaction at 25° > 5 % of the final colour. This condition is the opposite to that given in the preceding case and the interference in the colorimetric method cannot be due to purified reductone, reductic acid or the products obtained by the heat treatment of alkaline solutions of glucose because, if these were present, relatively higher titres with indophenol would be obtained after reaction with HCHO at pH 3·5. With the evidence so far available, it is impossible to state definitely the cause of the interference under these conditions in the colorimetric method.

Table 8. *A comparison of the results obtained by the Roe & Kuether and by the Lugg method applied to substances of unknown composition*

Substance	Ascorbic acid: Colorimetric method (mg./100 g.)	Ascorbic acid: Lugg's corrected method (mg./100 g.)	Indophenol titration method: Before H_2S (mg./100 g.)	Indophenol titration method: After H_2S (mg./100 g.)	'Ascorbic acid' at 25° in colorimetric method (%)	Relative titration values obtained with Lugg's method at pH 1·5 and 3·5	Remarks
Chocolate	21·4	22·4	23·8	26·1	<5	$v_2 < v_1/6$	No significant amount of reductic acid, reductone or the products from heat-treated alkaline glucose is indicated
(containing added ascorbic acid)	21·2	21·4	21·9	24·4			
	21·8	22·8	23·2	25·1			
Canned potatoes:							
Liquor	11·0	10·4	11·5	11·5	<5	$v_2 < v_1/6$	,, ,,
Tissue	8·1	7·5	8·6	9·2			
Stored dried cabbage	22·1	34·1	25·8	50·0	>5	$v_2 > v_1/6$	Both methods indicate the presence of interfering substances similar in character to the above
Stored dried soup	6·6	7·9	11·5	20·7	>5	$v_2 > v_1/6$	
Canned meat and vegetables:							
Potato	2·0	2·5	3·5	3·9	<5	$v_2 > v_1/6$	Interference in the Lugg method due to substances other than the above
Meat	1·8	2·8	1·9	4·0	,,	,,	
Gravy	1·9	3·5	4·8	5·4	,,	,,	
Bean	1·7	1·6	2·4	2·8	,,	,,	
Carrot	1·9	2·2	3·0	3·4	,,	,,	

To illustrate the value of a comparison of the two methods we introduce Table 8, in which the analytical results of a number of substances, which on theoretical grounds might be expected to contain some interfering substances, are given. It will be seen in the first place that the values obtained by direct indophenol titration either before or after H_2S treatment are consistently higher than those obtained by the formalin and 2:4-dinitrophenylhydrazine methods; in some cases the difference is very great. Values obtained before and after treatment with H_2S are given because it is not easy in these cases to say which is correct, since any increase after treatment may be due to the presence of dehydroascorbic acid or to the formation of indophenol-reducing substances other than ascorbic acid. The second and most important conclusion from this experiment is that the comparison of the two methods throws some light on the character of the interfering substances. Thus in the case of the stored dried cabbage the interference is in all probability due to the presence of one or of a mixture of substances such as reductone, reductic acid and products similar to those obtained by heating alkaline glucose solutions, whilst in the case of the meat and vegetable ration there is an indication that an indophenol-reducing substance or substances not previously investigated are present.

From the above considerations it may generally be concluded that each one of the methods is likely to yield more accurate results than the direct titration in acid solution, and if used in conjunction with one another some qualitative indication of the type of interfering substances may be obtained. In spite of this far greater specificity obtained with the Lugg and with the Roe & Kuether methods with solutions or extracts of unknown composition, caution must be used in the interpretation of the results until further knowledge is available, even if the figures should agree with those given by direct indophenol titration in acid solution.

SUMMARY

1. *d*-Glucoascorbic acid, *d*-araboascorbic acid and 2:3-diketo-*l*-gulonic acid cannot be distinguished from *l*-ascorbic acid by the Roe & Kuether method.

2. *l*-Ascorbic acid, reductic acid, purified reductone, heat-treated $NaHCO_3$ and Na_2CO_3 solutions of glucose and heat-treated NaOH solutions of glucose generate after 15 min. at 25° 2·5, 92, 48, 66 and 45% respectively of the colour given by these substances in 3 hr. at 37°, i.e. the conditions of the Roe & Kuether method.

3. Corrections can therefore be made for any one of the above products when they are present with *l*-ascorbic acid, providing that the analogues of ascorbic acid and diketogulonic acid are absent. In unknown solutions, however, the error due to these substances can be reduced but not eliminated.

4. When Roe & Kuether's method is used in conjunction with Lugg's method an indication of the types of interfering substances may be obtained.

5. Caution is urged in the interpretation of the results obtained by Lugg's and by Roe & Kuether's methods.

One of us (J. R. P.) is indebted to the Medical Research Council for a whole-time grant. We wish to express our gratitude to Prof. E. J. King for advice in connexion with the construction of the Evelyn type photoelectric colorimeter, to Prof. E. L. Kennaway for the use of a 'Spekker' standard type absorptiometer, to Dr J. C. Broom, of the Wellcome Research Institute, for the use of a 'Spekker' sensitive type absorptiometer and to Prof. E. L. Hirst for a gift of *d*-araboascorbic and *d*-glucoascorbic acid. Our thanks are also due to Roche Products Ltd. for *l*-ascorbic acid.

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The Effect of Mycophenolic Acid on the Growth of *Staphylococcus aureus* in Heart Broth

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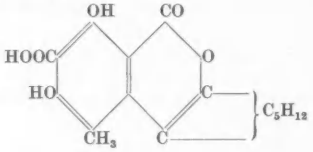
During an investigation of the production of antibiotics by fungi, Wilkins & Harris (1943) found that the culture liquid from *Penicillium brevi-compactum* Dierckx. 3568 inhibited the growth of *Staph. aureus*. The substance responsible for the inhibition was isolated in this laboratory and found to be mycophenolic acid (Florey, Gilliver, Jennings & Sanders, 1945). The molecular constitution of this substance had been studied by Clutterbuck & Raistrick (1933), who advanced the following structure for *nor*mycophenolic acid:



In mycophenolic acid itself one of the phenolic groups is methylated.

Florey *et al.* (1945) studied the biological properties of this antibiotic and noticed that when its activity against staphylococci was determined by the serial dilution method the titre varied greatly with the number of organisms used for the inoculum. To study this in more detail a quantitative investigation has been made of the effect of the antibiotic on the growth of staphylococci. It was soon apparent that the effect is a complicated one, involving a rapid adaptation of the staphylococci to growth in the presence of mycophenolic acid.

EXPERIMENTAL

Conditions of growth. Staph. aureus (H. strain, National Collection of Type Cultures (N.C.T.C.) no. 6571) was grown in aerated heart-extract broth at pH 7. The medium (20 ml.) was contained in pyrex glass tubes. The tubes were attached, by a ground-glass joint, to a hollow glass head-piece having an air inlet tube, an outlet and an opening with a ground-glass cap for taking samples (Fig. 1). The tubes and head-pieces were sterilized separately and fitted together aseptically, without grease, after sterile medium and the inoculum had been added to the tubes. The tubes were placed in a water-bath at 37° and a slow stream of sterile air, saturated with water-vapour at the same temperature, was blown through the medium. Samples were taken with long Pasteur pipettes.

Except in special circumstances, mentioned later, the staphylococci grew in the form of perfectly discrete cells.

Total counts. The total number of organisms was counted in a Neubauer haemocytometer, 0·1 mm. deep, with a grating of sixteen sets of sixteen squares. A drop of formalin and a trace of methylene blue were added to the sample,

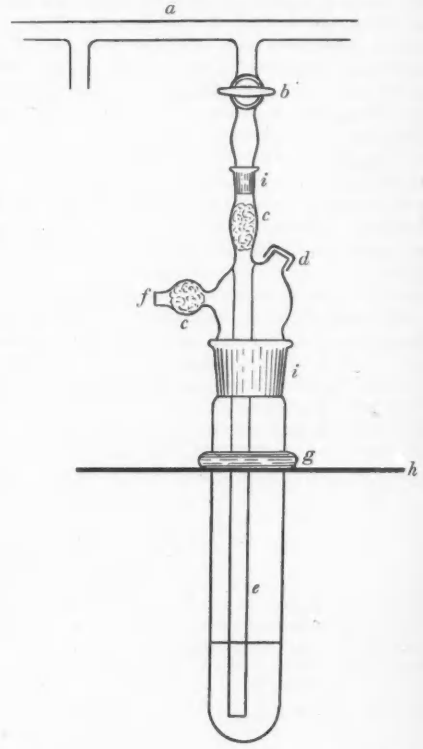


Fig. 1. *a*, manifold delivering sterile air; *b*, tap; *c*, cotton-wool plugs; *d*, opening for taking samples; *e*, air inlet; *f*, air outlet; *g*, rubber ring for supporting tube in rack *h*; *i*, ground-glass joints.

which was then diluted, if necessary, until it contained about 20×10^6 organisms/ml. The cells were allowed to settle for about 15 min. in the haemocytometer before being counted. The majority of counts was made on samples

containing more than 10×10^6 organisms/ml., a concentration at which a turbidity in the medium was just perceptible; no attempt was made to count samples having less than $4{\cdot}0 \times 10^6$/ml., corresponding to about one cell/small haemocytometer square or 256 in the whole grating.

If counts are made in N small haemocytometer squares giving a total Nn cells, the mean number of cells/square is n and the standard deviation of the distribution is $\sqrt{n}$. The standard deviation of the mean of N counts (standard error) $= \sqrt{(n/N)}$. Thus, if the whole haemocytometer grating is used to count a sample containing $4{\cdot}0 \times 10^6$ cells/ml. the standard error can certainly not be less than 6·2%. Counting in a haemocytometer 0·1 mm. deep presented no difficulty with staphylococci, which were easily seen and settled readily, but it is not suggested that the method would necessarily be satisfactory for counts of other species of bacteria.

Viable counts. The number of viable organisms was counted by the roll-tube method of Wilson (1922) or by spreading two drops of a suitably diluted culture, with the aid of a glass spreader, on the surface of three nutrient-agar plates. Dilutions were made in sterile tap water with calibrated dropping pipettes.

For counting samples containing relatively small numbers of organisms in the presence of the antibiotic the plate method was preferred. In these cases the roll-tube method gave rather lower values, possibly because it involved a smaller dilution of the antibiotic and also brought the latter into contact with the cells for a short time at 45°.

Inoculum. A 16 hr. culture in Lemco broth was made from a culture of *Staph. aureus* kept on an agar slope in the ice-chest. One loopfull of this culture was added to 5 ml. of heart broth in a 25 ml. Erlenmeyer flask and the flask shaken at 37° for 15–16 hr. The resulting culture contained about 3000×10^6 organisms/ml., at least 90% of which were viable. It was suitably diluted in sterile tap water and about five drops of the bacterial suspension, delivered by a calibrated dropping pipette, were used to inoculate 20 ml. of the medium in the tubes.

Growth curves

In all the growth curves $\log_{10} n_T$ or $\log_{10} n_v$ was plotted against t, where n_T was the total number of organisms/ml., n_v the number of viable organisms/ml., and t the time in minutes.

If there is a sudden transition from the lag phase to the logarithmic phase the common growth curve of a bacterial culture is given by

$$n = n_0 e^{k(t-L)} \text{ or } \log_{10} n = \log_{10} n_0 + \frac{k}{2{\cdot}303}(t-L),$$

where n_0 is the original number of organisms, n the number at time t, and L the length of the lag phase; k is a growth constant. The lag is defined here in accordance with this equation; it was measured by extrapolating the straight line representing the change of $\log_{10} n$ with t in the logarithmic phase until $n = n_0$ and reading the corresponding value of t.

The mean generation times (m.g.t.'s), or the times taken for dividing cells to double in numbers, were calculated from the slopes of the curves during the phase of logarithmic growth:

$$\text{m.g.t.} = \frac{2{\cdot}303 \log_{10} 2}{k}, \text{ where } k = 2{\cdot}303 \frac{d \log_{10} n}{dt}.$$

Normal growth

Growth in the batch of heart broth used for the first series of experiments is shown by curves 1, 2 and 3 in Fig. 2. The lag was 40–50 min. and did not vary significantly with the size of the inoculum. The m.g.t. was 28·2–28·7 min. The logarithmic phase of growth came to an end abruptly as the number of organisms approached 1000×10^6, which was defined as the stationary population (n_s): the rate of increase in numbers beyond this was relatively very slow. The pH of the medium, as measured by indicators, did not change by more than 0·5 unit before the stationary population was reached.

Growth in a 1 : 10^4 dilution of mycophenolic acid

The growth curves in the presence of mycophenolic acid at a dilution of 1 : 10^4 are shown by curves 4–8 in Fig. 2. It is convenient to consider the lag phase and logarithmic growth phases separately.

(1) *The lag phase*

The length of the lag phase, as estimated by extrapolation of the total counts during logarithmic growth, increased greatly as the size of the inoculum was decreased. That this extrapolation was justified was shown by making viable counts during the lag phase itself, the results of which are shown in Fig. 3. There is reasonably good agreement between the length of the lag determined by the two methods (Table 1). The viable counts also indicated that, at this concentration of the antibiotic, the estimated lag was due to non-division only and not partly to the killing of a significant number of cells before multiplication began.

Table 1. *Variation of length of the lag period with inoculum size*

(*Staph. aureus* in heart broth.)

Inoculum (n_0) no. of organisms (millions)	Length of lag by extrapolation of total counts (min.)	Inoculum (n_0) no. of organisms (millions)	Length of lag by viable counts (min.)
9·0	40	—	—
0·56	280	0·96	240
0·14	380	—	—
0·014	640	0·013	700
0·010	920	—	—

(2) *Phase of logarithmic multiplication*

Adaptation to growth in mycophenolic acid. In the presence of the antibiotic the m.g.t. was increased.

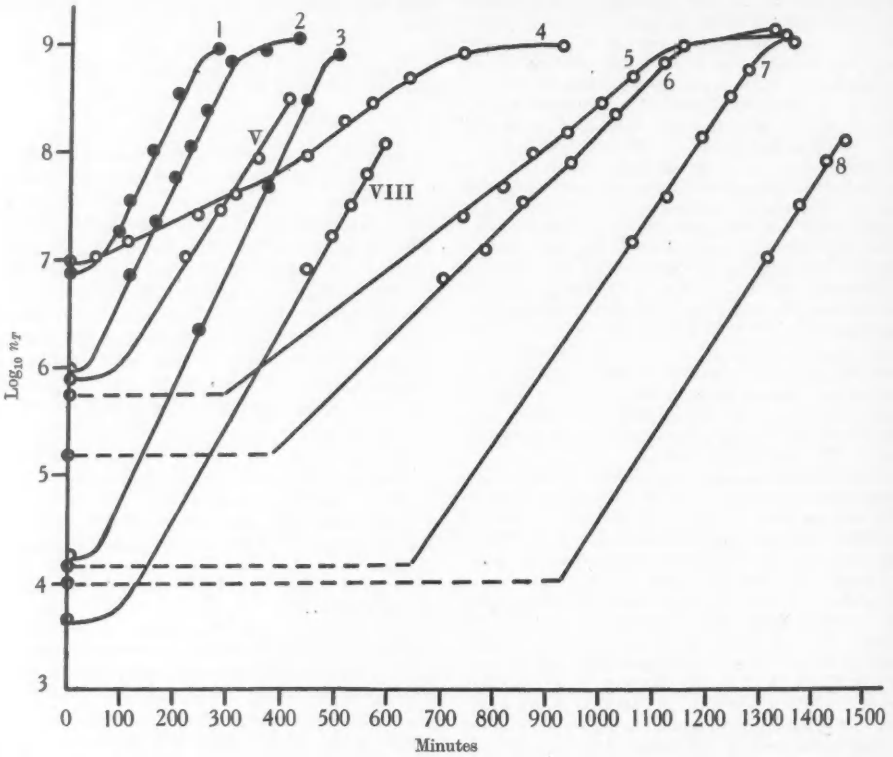


Fig. 2. Effect of inoculum size on growth. Curves 1, 2 and 3, growth in normal heart broth. Curves 4–8, growth in heart broth containing mycophenolic acid at a dilution of $1 : 10^4$. Curves V and VIII, subculture from 5 and 8 in the presence of mycophenolic acid ($1 : 10^4$).

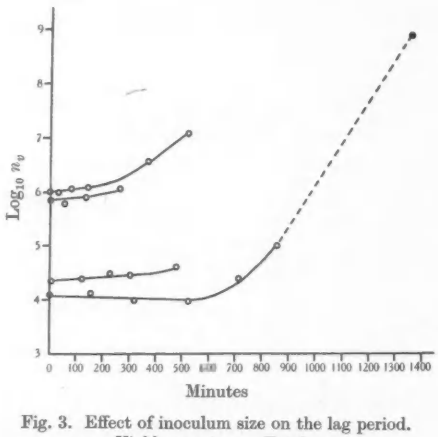


Fig. 3. Effect of inoculum size on the lag period.
o—o Viable counts. • Total count.

The curves (Fig. 2) representing the rates of multiplication present three points of interest: (1) The rate of division when total counts were first possible (n = approx. 10×10^6) increased as the inoculum used was made smaller. (2) Except with the

Table 2. *Variation of the rate of division with inoculum size in the two parts of the logarithmic growth phase*

(*Staph. aureus* in heart broth containing mycophenolic acid ($1 : 10^4$).)

Inoculum (n_0) no. of organisms (millions)	m.g.t.* first phase $\log_{10} n = 7{\cdot}2$–$7{\cdot}7$ (min.)	m.g.t.* second phase $\log_{10} n = 8{\cdot}2$–$8{\cdot}7$ (min.)
9·0	137	83
0·56	80	70
0·14	63	58
0·014	41	41
0·010	39	39

* m.g.t. = mean generation time.

smallest inocula the growth curves were clearly composite, the rate of division increasing fairly suddenly after n had reached approximately 100×10^6. Growth in the separate parts of the curves was nearly logarithmic. (3) The size of the stationary population was not changed significantly by the antibiotic. The data relating to (1) and (2) are summarized in Table 2.

The changes in the m.g.t.'s during logarithmic growth showed that the staphylococci were rapidly becoming adapted to multiplication in the presence of mycophenolic acid. The effect of this adaptation was clear from a study of the entirely different growth curves of subcultures.

Growth and stability of the adapted staphylococci. Curves V and VIII (Fig. 2) represent the growth of inocula taken from the cultures recorded in curves 5 and 8 (Fig. 2) a few hours after these had reached their stationary concentrations. In both cases the lag was short (about 90 min.) and the m.g.t. low (39 and 35 min.).

After subculturing culture VIII twice more in heart broth containing a $1 : 10^4$ dilution of mycophenolic acid there was little, if any, increase in adaptation. The lag was then 80 min. ($n_0 = 1{\cdot}0 \times 10^6$) and the m.g.t. 36 min.

After subculturing culture VIII twice through heart broth not containing the antibiotic there was no significant loss in adaptation, the lag being 80 min. ($n_0 = 1{\cdot}0 \times 10^6$) and the m.g.t. 37 min.

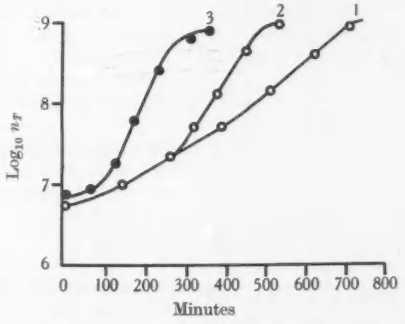


Fig. 4. Adaptation to growth in presence of mycophenolic acid (1 in 10^4). Curve 1, primary growth curve. Curve 2, subculture from 1. Curve 3, growth in normal heart broth.

The course of adaptation to a $1 : 10^4$ dilution of mycophenolic acid when starting from a fairly large inoculum ($n_0 = 6{\cdot}0 \times 10^6$) is indicated by the curves in Fig. 4. Curve 1 shows the primary growth and curve 2 the curve obtained with an inoculum taken from culture 1 a few hours after the latter had reached its stationary population. The adaptation consisted in a shortening of the time during which the slower rate of growth occurred and in a transition to a faster rate of division than the corresponding rate in the primary culture. In the absence of the antibiotic the adapted staphylococci grew at the same rate as the normal organisms.

Effect of adding mycophenolic acid during logarithmic growth. In Fig. 5 curves 2 and 3 show the effect on growth of mycophenolic acid when added to give a dilution of $1 : 10^4$ at the beginning (curve 2) and in the middle of the logarithmic phase (curve 3). The rate of division decreased immediately, the m.g.t. being approximately doubled. After a short time this was followed by a further decrease to a very slow rate of growth before adaptation began.

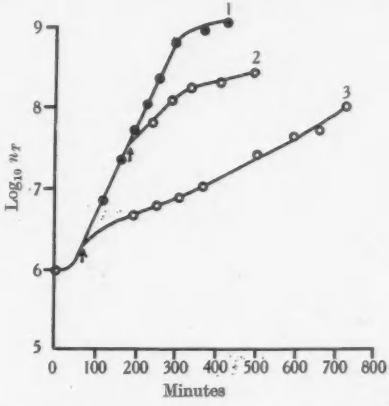


Fig. 5. Addition of mycophenolic acid (to a dilution of $1 : 10^4$) at two stages of logarithmic growth.

Effect of culture-filtrates on growth in the presence of mycophenolic acid. The dependence of the length of the lag phase on the inoculum size in the presence of mycophenolic acid made it desirable to investigate the effect on growth of filtrates from young cultures.

(1) An inoculum of $1{\cdot}0 \times 10^6$ cells/ml. was allowed to grow in normal heart broth to about 20×10^6 and the culture then centrifuged clear and sterilized by filtration through a Gradacol membrane. 5 ml. of heart broth were added to a tube containing 15 ml. of the filtrate and the mixture inoculated so that it contained $0{\cdot}8 \times 10^5$ organisms/ml.; a similar inoculum was used for a control tube containing 20 ml. of heart broth; mycophenolic acid was added to both media to give a dilution of $1 : 10^4$. Viable counts were made at intervals on samples from the two tubes. In the normal heart broth the number of organisms remained practically constant for 500 min. In the filtrate a very slow rate of growth began but stopped after about one division. When the inocula grew up the organisms in the filtrate

reached a count of 100×10^6, 30 min. before those in normal heart broth. The final effect of the filtrate was therefore rather small.

(2) An inoculum of $5{\cdot}0 \times 10^6$ cells/ml. was allowed to grow to about 50×10^6 in a $1 : 10^4$ dilution of mycophenolic acid in medium, at which stage it had almost reached the point at which transition occurred from the slower to the faster rate of growth. The culture was centrifuged and filtered. Growth was then compared in the filtrate and in normal heart broth containing the same concentration of the antibiotic. (It had been shown that mycophenolic acid was not inactivated during growth of the staphylococci.) The growth curves are shown in Fig. 6. In normal heart broth (curve 1) there was

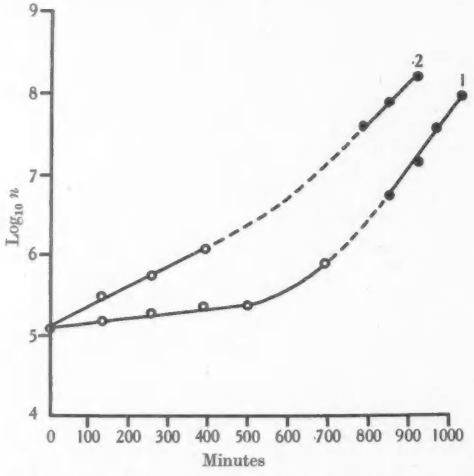


Fig. 6. Abolition of the lag phase by filtrate from a young culture. Curve 1, growth in heart broth containing mycophenolic acid ($1 : 10^4$). Curve 2, growth in filtrate from a young culture containing mycophenolic acid ($1 : 10^4$). o—o Viable counts. •—• Total counts.

a lag period of 600 min.; in the filtrate (curve 2) division began immediately, with a m.g.t. of 125 min. which was maintained until transition occurred to a faster rate of growth. Culture (2) reached a count of 100×10^6, 170 min. before culture (1). There was thus no doubt about the effect of the filtrate in abolishing the lag period.

Effect of concentration of mycophenolic acid. In Fig. 7 curves 1–5 represent growth from inocula of similar size (n_0 = approx. $5{\cdot}0 \times 10^6$) in the presence of concentrations of mycophenolic acid increasing from a dilution of $0{\cdot}25 : 10^4$ to a dilution of $5{\cdot}0 : 10^4$. With the apparent exception of curve 5 all the curves are composite, consisting of a relatively short lag, a period of slow division and a period of more rapid division. In the case of the highest concentration (curve 5) the dotted line indicates that the long lag represented by the number of organisms counted does not give a true picture of the course of events. During this period morphological changes occurred, resulting in the adherence of some of the cells to the sides of the tube; the numbers counted in the supernatant liquid, which contained for the

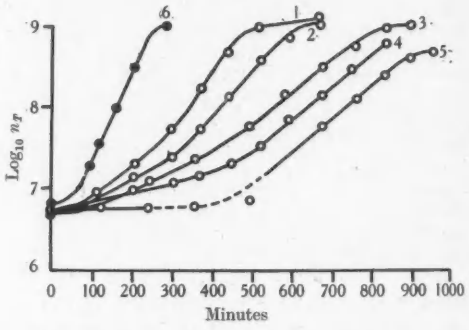


Fig. 7. Effect of the concentration of mycophenolic acid on growth. Curve 1, 0·25 in 10^4; curve 2, 0·5 in 10^4; curve 3, 1·0 in 10^4; curve 4, 2·5 in 10^4; curve 5, 5·0 in 10^4; curve 6, control, growth in heart broth without mycophenolic acid.

most part discrete cells, therefore gave low values in terms of bacterial mass. When more rapid growth set in, this was normal in character and most of the adhering cells disappeared. The stationary population ($n_s = 550$) was lower than that reached in the presence of lower concentrations of the antibiotic ($n_s = 1100$).

The rate of division in the first phase decreased, but by no means linearly, with the increasing concentration of the antibiotic. The rate of division in the second phase decreased to a value, about 0·4, as great as that of a normal culture, at which it remained practically constant during a further fivefold increase in concentration of antibiotic. This is shown graphically in Fig. 8. In the presence of a $5{\cdot}0 : 10^4$ dilution of mycophenolic acid the numbers of viable organisms also were counted at intervals during the first 700 min.; there was good agreement between the viable and total counts.

To see whether the highest concentration of antibiotic was approaching one at which it would exert a bactericidal action, viable counts were made at intervals for 420 min. on samples from a tube inoculated with 10×10^6 organisms/ml. and containing a $1 : 10^3$ dilution of mycophenolic acid. There was a very slow increase in the number of cells which, at the end of this time, reached 20×10^6. A corresponding change occurred in the total num-

ber of organisms present. Thus, under these conditions, 0·1 % mycophenolic acid exerted no detectable lethal effect on the cells.

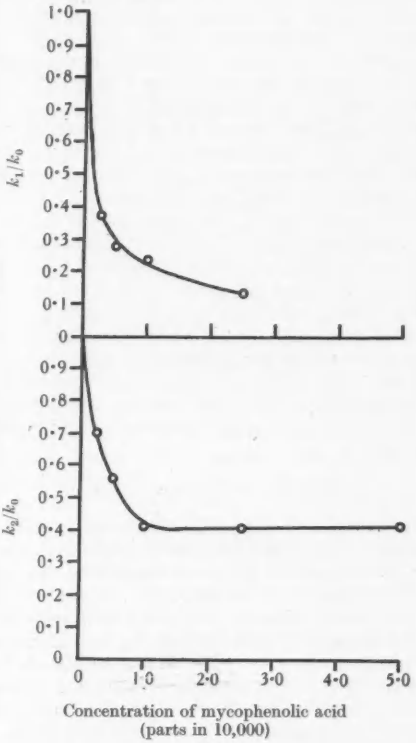


Fig. 8. Variation of the ratio of the growth constants with various concentrations of mycophenolic acid to the growth constant in normal heart broth.

Morphological changes during growth in the presence of mycophenolic acid

The first series of measurements on growth in the presence of a 1 : 10^4 dilution of mycophenolic acid (Fig. 2) was made using a single batch of heart broth, which was kept sterile in 100 ml. bottles at room temperature. After 2 months, although the lag, m.g.t. and n_s of the staphylococci grown in this medium were not measurably different from their original values, morphological changes, consisting in the formation of clumps of cells which had a tendency to adhere to the walls of the vessels, occurred when growth took place in the presence of a 1 : 10^4 dilution of mycophenolic acid. As these changes continued to occur when the staphylococci were taken from different agar slopes, while growth was normal in a fresh sample of heart broth in the same concentration of mycophenolic acid, it was concluded that alterations which had taken place in the medium on standing were responsible for the abnormal mode of growth. This medium is subsequently referred to as 'old heart broth' (O.H.B.).

The physical properties of the clumps of cells made it impossible to investigate quantitatively their numbers and sizes, but the following facts were noted: (1) Clumping only occurred after growth had begun. At the end of the lag period about one division took place in an apparently normal manner before any morphological changes appeared. The degree of clumping then increased to a maximum and subsequently decreased as the growth cycle proceeded. (2) The morphological changes were more pronounced when growth occurred in filtrate, to which mycophenolic acid had been added, from young cultures in O.H.B. When small inocula were used the filtrate greatly shortened the time which elapsed before clumping began, a result which might be expected if the filtrate abolished the lag period. (3) After two subcultures in O.H.B. containing mycophenolic acid the staphylococci had become trained to grow in this medium without any morphological changes taking place. The growth curve was then very similar to that of the staphylococci which had become adapted to mycophenolic acid by growth in a sample of heart broth in which clumping did not occur at all.

Results

The main facts which emerge from a study of the growth curves are: (1) When *Staph. aureus* (H. strain, N.C.T.C. no. 6571) is grown in normal heart broth the short lag phase is not altered appreciably by changes in the inoculum size. (2) Mycophenolic acid lengthens the lag phase and the mean generation time; at a dilution of 1 : 10^4 it has little effect on the size of the stationary population. (3) As the inoculum size is made smaller the effect of mycophenolic acid on the lag phase is greatly increased. The reverse effect is found on the m.g.t.; within certain limits the smaller the inoculum the greater the rate of division when logarithmic multiplication has set in. (4) Filtrate from a young culture grown in the presence of mycophenolic acid abolishes the long lag phase of small inocula. Growth is also stimulated by filtrate from a young normal culture. (5) During multiplication in the presence of the antibiotic a slow rate of division may be superseded by a faster one, resulting in composite growth curves. (6) The growth rates in both parts of the growth curve are decreased, but not linearly, by increasing the concentration of the antibiotic; the faster growth rate reaches a limiting value below which it does not fall while the concentration of antibiotic is increased fivefold. (7) During

growth a rapid adaptation of the staphylococci occurs which may be almost complete in one culture cycle. The adapted organisms are characterized by a short lag and by a high rate of division in the presence of the antibiotic. Reversal of the adaptation, if it occurs, is comparatively very slow. (8) Minor alterations in the medium can result in gross morphological changes during growth in the presence of mycophenolic acid, but the staphylococci are rapidly adapted to grow without these changes occurring. The changes are accentuated by filtrate from a young culture grown in the same medium in the absence of the antibiotic.

DISCUSSION

The inoculum size in the serial dilution method of estimating antibacterial activity

Within certain limits the variation of dilution test readings with inoculum size does not in itself imply that the size of the inoculum has any effect on either the lag or the rate of division. It is obvious that, provided two inocula differ sufficiently in size, one can be fully grown before any growth is visible from the other, so that on reading the tubes *within a given time* there would be a clear-cut difference in turbidity.

Suppose n_{01} is the large and n_{02} the small inoculum. When n_{01} has just grown to its stationary population n_s, n_{02} has grown to a value n_2, where

$$\log n_s - \log n_{01} = \log n_2 - \log n_{02} = k\,(t-L),$$

or

$$n_{01}/n_{02} = n_s/n_2.$$

The condition that at any time there should be full growth in one tube and no visible growth in the other is that $n_2 < 10 \times 10^6$ or $n_{01}/n_{02} > n_s/10 \times 10^6$. If n_s is 1000×10^6 this condition is satisfied when $n_{01}/n_{02} > 100$.

With a hundredfold dilution of the inoculum this state of affairs holds only momentarily. With a thousandfold dilution, however, it is maintained during a time T sufficient for n_{02} to increase tenfold. Therefore $T = \text{m.g.t.}/0{\cdot}3$. If a given concentration of an antibacterial substance increased the m.g.t. from 30 min. to, say, 100 min., $T = 5{\cdot}5$ hr. If n_{01} and n_{02} were 1000×10^3 and 1×10^3 respectively the larger inoculum would reach its stationary population in 1000 min. and the smaller inoculum its visible population in 1333 min. Suppose, as has been shown in some cases (e.g. Dagley & Hinshelwood, 1938), that the growth constant k varied linearly with the concentration of the antibacterial substance, i.e. $k = k_0 - dC$, where C is the concentration and d is a constant. Then it is easily calculated that, if the concentration of the antibacterial substance were halved, the smaller inoculum would reach its stationary population in 920 min. If the concentration of antibacterial substance were doubled, k would appear as a negative quantity, and there would be no growth at all. Thus, in a twofold dilution test carried out under these conditions, and read between 16·5 and 22 hr., complete inhibition of growth would be shown in one extra tube in the serial dilutions in the case of the smaller inoculum. If the effect of the inoculum size were considerably greater than this it would be expected either that growth depended in some specific manner on the size of the inoculum or that the rate of growth was altered relatively little by changes in the concentration of the antibacterial substance. Both these factors were operative in the case of mycophenolic acid. Inspection of curve 7, Fig. 2, and curve 5, Fig. 7, shows that, between 15 and 17 hr. after inoculation, there was no visible growth from the smaller inoculum while an inoculum 360 times as large had reached its stationary population in a fivefold greater concentration of mycophenolic acid. The determining factors here were the considerable lengthening of the lag phase as the inoculum size decreased and the adaptation of the cells to grow rapidly even in relatively high concentrations of mycophenolic acid.

Adaptation and natural selection

In the vast literature on bacterial variation widely different suggestions have been made to account for the changes which can occur within the same bacterial species (cf. Hadley, 1937). Apart from the detailed mechanism of such variations, however, a difference in properties between a given culture and its parent must have arisen in one or both of two ways: (1) by natural selection of cells from a heterogeneous population; (2) through changes induced in the cells themselves.

Some 'adaptive' enzymes not directly concerned with growth appear to be formed in the absence of cell division (Stephenson & Yudkin, 1936; Knox & Pollock, 1944) which would preclude their emergence through a natural selection. Hinshelwood (1944) has stated a number of arguments in favour of the view that, at least in those cases in which the variation consists in the adaptation of bacteria to grow in new media or in the presence of drugs, the essential role is played by a modification of the enzyme systems of the cells. It is thought that the behaviour of *Staph. aureus* to mycophenolic acid is to be interpreted most satisfactorily from this point of view.

It is clearly possible to obtain a rough approximation to any one of the composite curves in Fig. 2 by superimposing curves calculated for given inocula of two sets of organisms growing independently with appropriate lags and m.g.t.'s. The slope of such a curve at any moment is given by

$$d\ln n/dt = k_1 n_1/n + k_2 n_2/n,$$

where n is the total number of organisms and n_1 and n_2 the numbers of cells of the separate substrains, whose growth constants are k_1 and k_2. Plotting $ln\ n$ against t never gives a really straight line, but in general, as the faster-growing strain overtakes the slower-growing one, the slope of the curve may change from a value not far from k_1 to a value not far from k_2 during three or four divisions. To obtain a reasonable approximation to all the curves in Fig. 2, however, on the assumption that these represent only a selection of strains of different sensitivity, it would appear necessary to assume the presence of several substrains whose lags varied in a complicated manner with the inoculum size. Moreover, suppose curve 8 resulted from the least sensitive substrain multiplying while the rest of the organisms remained in the lag phase. From curves 5 and 8 this substrain would be assigned a short lag of about 80 min. It would therefore have apparently grown up from an initial value of 0·12 cells/tube. The chance that a given tube would not contain one cell of this substrain would be $e^{-0·12}$ or 9 to 1; while if it did contain one cell the experimental and synthetic curves would no longer fit. It would not seem plausible to assume that the adaptation depended solely on natural selection.

Two kinds of change in the enzyme systems of bacterial cells might be responsible for an adaptation to growth in the presence of an antibiotic. First, enzymes concerned with normal or alternative growth mechanisms might increase in quantity/cell. Secondly, an enzyme might be produced which inactivated the antibiotic itself. A change of the latter type was not concerned in the adaptation to mycophenolic acid. Filtrates from cultures which had reached their stationary populations from small inocula in the presence of the antibiotic showed no decrease in antibacterial activity when tested by the ring method (Heatley, 1944).

The lag phase

A decrease in the length of the lag phase with an increase in the inoculum size means that the individual cell is influenced through the medium by its neighbours; it clearly suggests that a substance diffusing from the cells is concerned with the ending of the lag.

It would also be possible, if less likely, that an inhibitor diffused *from* the medium to the cells where it was destroyed. The two cases would be sharply differentiated by attempts to concentrate the diffusible substance.

There are many recorded instances of growth depending on the size of the inoculum. Thus Mueller (1939) found that *C. diphtheriae* grew well when seeded thickly on a solid medium, while when it was thinly sown growth either did not occur at all or else only after a prolonged lag. Later Cohen, Snyder & Mueller (1941) obtained evidence that the poor growth from a light inoculum might be due in part to absence of oleic acid. Hoffmann & Rahn (1944) found that crystal violet increased the lag phase of *Esch. coli* and *Streptococcus lactis* and that this increase was greater the smaller the size of the inoculum. Lodge & Hinshelwood (1943) found that when *Bact. lactis aerogenes* was grown in an artificial medium with ammonium sulphate as the nitrogen source, the lag was greatly affected by the age of the inoculum. With very young cultures the lag was considerable, but as the age increased it rapidly fell to a minimum and finally again increased. *The first or 'early lag'* increased as the inoculum size was made smaller; it was abolished by filtrate from an actively growing culture which was approaching the age at which it showed the minimum lag, proving that a diffusible lag-removing substance was present in the medium. Lag was thought to end when this substance reached a threshold concentration in the cells. Later it was found that 'early lag' was increased by the presence of proflavine, methylene blue and sulphonamides and that this effect of these substances was antagonized by sterile filtrate (Davies & Hinshelwood, 1943; Davies, Pryce & Hinshelwood, 1944). It was therefore concluded that, *inter alia*, these inhibitory substances hindered the synthesis or utilization of the diffusible substance which abolished the lag.

The part played by the inoculum size under the conditions used in the present work is similar but not identical. Thus, the lengthening of the lag phase appears only in the presence of mycophenolic acid and not with small inocula in normal cultures. If the reasonable assumption be made that the first effect of the antibiotic is to damage but not destroy the normal growth mechanism this might be explained in several ways: (1) A diffusible growth-intermediate might be supplied in great excess by the normal cells so that even after considerable dilution it would still be present in optimum amounts. If the antibiotic interfered with the production of the intermediate this might no longer be true. (2) The antibiotic might change the permeability of the cell membranes, allowing the diffusion of a substance normally retained in the organisms. (3) The antibiotic might affect the rate of utilization of a diffusible intermediate. Suppose, as a very simple case, that the consecutive reactions

$$A \underset{k_1}{\rightarrow} B \underset{k_2}{\rightarrow} C$$

occur in the cells and that substance B is diffusible. If, in the normal cell, the velocity constant k_2 is much greater than k_1 the concentration of B will remain low and very little may be lost by diffusion during the conversion to C; the over-all reaction rate will be governed by k_1. If now k_2 be decreased

by the action of an antibiotic, B will accumulate and for a time after inoculation the rate of formation of C in the cells may be greatly dependent on the inoculum size. An explanation of a similar kind would apply if the antibiotic altered the dissociation constant of a coenzyme and its protein carrier.

The consequences of these hypotheses are different; hypothesis (1) implies that the diffusible substance would be present in larger amounts in filtrate from a young normal culture than in filtrate from a culture grown in the presence of the antibiotic, while (2) and (3) imply the opposite. Further work is necessary to estimate the relative quantities of the diffusible substance in the two filtrates. Although the gross effect of the normal filtrate was much the smaller of the two it may have been complicated by morphological changes (see below). At present it is reasonable to conclude that the primary action of mycophenolic acid on the staphylococci consists partly in hindering the utilization or synthesis of a diffusible growth-intermediate produced in the cells. With large inocula the substance reaches a sufficient concentration in the cells to allow slow multiplication to begin very soon, but with small inocula this concentration is not attained and the first slow growth is replaced by a lag period.

The phase of logarithmic growth and the mechanism of adaptation

The curves representing the multiplication of the staphylococci from large inocula in the presence of mycophenolic acid show qualitatively some striking similarities to the curves obtained by Davies & Hinshelwood (1943) and by Lodge & Hinshelwood (1944) while investigating the adaptation of *Bact. lactis aerogenes* to growth in the presence of sulphonamides and to certain changes of medium. In all these cases there is a transition point at which a faster rate of growth replaces a slower one and the adaptation consists, partly at least, in a shortening of the period which elapses before the transition point is reached. Moreover, in the first two cases the m.g.t.'s fell with increasing concentration of the antibacterial substance, not to zero, but to limiting values. The composite curves obtained with *Bact. lactis aerogenes* were interpreted as showing the operation of alternative modes of growth, one less efficient but readily mobilized, the other more efficient, when in action, but only mobilized after a lag phase. This would appear to be the most satisfactory way of interpreting the composite curves obtained in the present investigation.

The effect of the concentration of mycophenolic acid on the growth constants could be expressed formally by an equation of the type

$$k = k_0 - \frac{\alpha C}{1+\beta C},$$

where C is the concentration and α and β are constants. $\frac{\alpha C}{1+\beta C}$ is the Langmuir expression for the variation with concentration of the amount of substance adsorbed on a surface. In these terms the constant $\left(1-\frac{\alpha}{\beta k_0}\right)$ would be a measure of the proportion of the 'growth patterns' in the cells untouched by the antibiotic.

If the primary curves obtained with large inocula in the presence of mycophenolic acid are compared with those of the adapted organism it is evident that one result of adaptation is a greatly increased rate of growth and another is the decrease in the time interval before the increased rate begins. If the increased rate of division is itself considered as an adaptation, a comparison of curves from inocula of different sizes indicates that this is a more important function of the time that the organisms have been in contact with the antibiotic than of the number of divisions that have occurred in its presence. Once logarithmic growth begins from a small inoculum, after a lag period, it does not proceed through well-defined phases with successively faster growth rates connected by transition points, but attains fairly rapidly its maximum rate. This lends support to the view that alternative modes of growth are involved in the adaptation and that this is not merely an expansion of the original mechanism. It could be assumed that during the long lag period, in which the normal mechanism is unable to function, intermediates are being formed which finally set the alternative mechanism in action. Such a picture of the adaptation is, however, oversimplified. There are not only two rates of growth but a whole series, depending on the length of the lag which precedes the onset of logarithmic multiplication. It appears as though a number of intermediate states were stable for a time in the cell, in which both mechanisms were contributing to growth in varying proportions.

The adaptation to mycophenolic acid was not easily reversible. In the literature of bacterial variation there are many instances recorded in which an adapted or variant organism is very stable and many others in which it reverts easily. It has often been assumed that entirely different processes are involved in the two cases, but the differences are generally of degree rather than of kind and can hardly serve in themselves as a satisfactory basis for classification. If an adaptation consists in the development of an alternative mode of growth there is no reason to expect that it should be easily reversible, provided the new mechanism competes in efficiency with the old, under normal conditions. The staphylococci adapted to mycophenolic acid grew in the presence of the antibiotic with a facility approaching that of normal growth and in its

absence at a rate indistinguishable from the normal. It is therefore not surprising that reversion did not rapidly occur.

The way in which the enzyme balance in bacterial cells may change is at present largely a matter of speculation. Yudkin (1938) considered enzymes to be in equilibrium with precursors, the combination of an enzyme with a substrate or inhibitor leading to the formation of more enzyme from the precursor. Davies & Hinshelwood (1943) have suggested that bacterial protein is built up from suitable fragments by a process similar to crystallization. The type of reaction they envisage is formulated:

Substrates + enzyme = extended enzyme
+ products used in other syntheses.

When the cells are undergoing steady multiplication the increase in mass of a given enzyme must follow the same law as cell growth, i.e. $E = E_0 . e^{kt}$, where k is the growth constant. The physico-chemical implications of disturbing simple systems of this sort at specific points by the action of antibacterial substances have been worked out in some detail (Davies *et al.* 1944; Lodge & Hinshelwood, 1944). It has been shown that in such cases adaptation may occur automatically and rapidly, either by expansion of one enzyme system in relation to another or by readjustment of the balance in a single system. Under optimum conditions adaptation is almost complete when the new bacterial mass outweighs the old and if certain simple conditions are fulfilled it is not easily reversible. There does not appear to be anything in the behaviour of *Staph. aureus* to mycophenolic acid which is incompatible with this attractive theory. Processes which are more complicated but fundamentally of this nature may well play an important part in the adaptation of bacteria to the presence of antibacterial substances and to changes of medium, though it is not likely that they can account for the whole phenomenon of bacterial variation.

Morphological changes

There are obvious resemblances between the occurrence of the morphological changes in the staphylococci and the formation of snake forms in cultures of *Bact. lactis aerogenes* (Hinshelwood & Lodge, 1944). The mean length of the abnormal cells of *Bact. lactis aerogenes* increased to a maximum at about the middle of the logarithmic growth phase and then decreased. Their formation was induced by changes in media or by the addition of certain antibacterial substances and was stimulated by the addition of culture filtrates; the abnormal forms disappeared on 'training'. These facts resemble so closely those relating to the clumped forms of staphylococci that it would seem likely that similar factors are operating in the two cases.

Wyckoff (1934) concluded from micro-motion pictures of bacteria undergoing multiplication that growth and division depend on different factors which may vary independently. Hinshelwood & Lodge (1944) considered the phenomenon of snake-like cell formation in terms of two independent factors, one diffusible and responsible for elongation, one retained by the cells and responsible for division. Snake forms were thought to appear whenever, owing to changes in the medium or the action of antibacterial substances, the production of these factors was put out of balance. When this balance was restored reversion to normal growth took place. How far this simple hypothesis is capable of explaining the abnormal growth forms of the staphylococci it is inadvisable to decide without further information about the nature of the clumped cells; the physical properties of these clumps suggest that other factors may also be involved.

SUMMARY

1. When *Staph. aureus* (H. strain, N.C.T.C. no. 6571) was inoculated into heart broth containing mycophenolic acid the period of lag, preceding logarithmic growth, lengthened greatly as the size of the inoculum was made smaller. The long lag with a small inoculum was abolished when heart broth filtrates from young cultures were used as a growth medium. It is considered that a diffusible growth intermediate produced by the cells is concerned with the ending of lag and that the primary action of mycophenolic acid consists partly in hindering the synthesis or utilization of this substance.

2. The staphylococci were rapidly adapted to grow more easily in the presence of the antibiotic; this could be manifested during multiplication by a transition from a slower to a faster rate of division. The adaptation consisted in a shortening of the time which elapsed, after inoculation, before the rapid rate of growth set in; the adapted organism multiplied quickly after only a short lag even from a small inoculum. This adaptation was not easily reversible. The most likely mechanism of the adaptation is thought to consist in a modification of enzyme systems of the bacterial cells, resulting in the development of an alternative mode of growth.

3. The rates of division during logarithmic growth varied with the concentration of mycophenolic acid in a manner which suggested that a certain proportion of the growth mechanism was relatively insensitive to the antibiotic.

4. Minor alterations in the medium led to morphological changes during growth in the presence of the antibiotic, consisting in a failure of the dividing cells to separate in the normal manner. The organism was easily trained, by subculturing, to grow without these changes occurring.

It is a pleasure to acknowledge the expert technical assistance of Miss Ruth Callow.

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Metabolism of Acetoacetate in Animal Tissues. 1

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The utilization of acetoacetic acid in animal tissues is commonly ascribed to two types of metabolic processes: reduction to β-hydroxybutyric acid and oxidation to CO_2 and water. The reduction and its reversibility was first observed by Friedmann & Maase (1910) and Wakeman & Dakin (1910). The occurrence of an oxidative breakdown became established when Magnus-Levy and Knoop showed that acetoacetic acid is a normal intermediate in the decomposition of fatty acids. It was further demonstrated in conclusive experiments by Snapper & Grünbaum (1927). This pioneer work was later confirmed and amplified by many workers, especially when the tissue slice technique was applied (Jowett & Quastel, 1935; Quastel & Wheatley, 1935; Edson & Leloir, 1936). Reference to details of this work will be made later in this paper in connexion with our own findings.

As yet, relatively little is known about the intermediary steps in which acetoacetate disappears. Krebs & Johnson (1937) showed that the reduction can be brought about by dismutation with α-ketonic acids:

$$\text{acetoacetate} + \alpha\text{-ketoglutarate} + H_2O \rightarrow \beta\text{-hydroxybutyrate} + \text{succinate} + CO_2,$$

$$\text{acetoacetate} + \text{pyruvate} + H_2O \rightarrow \beta\text{-hydroxybutyrate} + \text{acetate} + CO_2.$$

As for the mechanism of oxidation, Weil-Malherbe (1938) excluded α-hydroxyacetoacetate as an intermediate. Hoff-Jorgensen (1940) reported that erythro-1:2-dihydroxybutyrate is rapidly destroyed after intravenous injection, whilst little or none of the threo-form is attacked under the same conditions. Weil-Malherbe (1938), in contrast, found that the threo-form increases the O_2 uptake of, and is removed by, kidney cortex, whilst the erythro-form was inactive. Kleinzeller (1943) found that crotonate, vinylacetate, γ-hydroxybutyrate and *dl*-2:3-dihydroxybutyrate are oxidizable in kidney cortex. The substances which are metabolized are possibly, but not necessarily, normal intermediates; further experiments will be required to decide whether they are normal metabolites.

Breusch (1943) and apparently independently Wieland & Rosenthal (1943) recently suggested that the $—CH_2.CO_2H$ grouping of acetoacetate is transferred as a whole to oxaloacetate, resulting in the formation of citric acid:

$$CH_3.CO.CH_2.CO_2H + CO_2H.CO.CH_2.CO_2H + H_2O \rightarrow \text{citric acid} + \text{acetic acid (Breusch, 1943)}, \quad (1)$$

$$CH_3.CO.CH_2.CO_2H + 2CO_2H.CO.CH_2.CO_2H + H_2O \rightarrow 2 \text{ citric acid (Wieland \& Rosenthal, 1943)}. \quad (2)$$

These schemes attempt to explain the observation that under some conditions oxaloacetate and acetoacetate together form more citric acid than oxaloacetate alone.

In the present investigation an effort is made to examine as fully as possible the chemical changes which accompany the disappearance of acetoacetate from animal tissues. It is already known from previous work (Jowett & Quastel, 1935; Breusch, 1943) and was soon confirmed by our own experiments

that the removal of acetoacetic acid is in some tissues linked up with the metabolism of the C_4 dicarboxylic acids, especially fumarate, malate and oxaloacetate. Preliminary work indicated that the chief chemical changes associated with the removal of acetoacetate concern, apart from β-hydroxybutyrate, the di- and tricarboxylic acids of the tricarboxylic acid cycle. We determined therefore the changes in the concentrations of the following 15 substances: acetoacetate, β-hydroxybutyrate, the eight acids of the tricarboxylic acid cycle, pyruvate, lactate, α-hydroxyglutarate, O_2 and CO_2. In some experiments most of the above metabolites were estimated, in others only a few.

EXPERIMENTAL

Tissue material

Minced versus sliced tissues. Preliminary experiments on sheep kidney cortex showed that the rates of removal of acetoacetate in sliced and in minced tissue are under certain conditions of the same order of magnitude. We preferred to use minced tissues for most experiments because relatively large quantities of tissues—between 10 and 20 g. fresh weight—were frequently needed for the many different analyses. To slice these quantities would have required too much time. It is possible to mince 20 g. of tissue within a few minutes, whereas slicing the necessary amount would have taken at least 30–40 min. and might have resulted in loss of enzyme activity.

Choice of tissue. Mammalian heart muscle was found to have the highest rate of acetoacetate removal, $Q_{ac.-ac.}$ reaching values up to −30. Kidney cortex was about half as active as heart; in other tissues the rate of acetoacetate metabolism was very much lower. In pigeon breast muscle, for example, the rate is only of the order of magnitude of the non-enzymic decomposition and its accurate measurement is therefore difficult.

Most experiments were carried out on sheep heart muscle obtained from the abattoir. The animal was electrically stunned and bled through the carotids. Immediately afterwards the sternum was sawn apart and the heart, still beating, removed and placed in a jar containing ice and iced water. It was then transported to the laboratory which it reached within 20 to 40 min. The rapid removal of the heart from the animal and efficient cooling were essential for the preservation of the metabolic activities. The enzymes in heart muscle, like those of skeletal muscle, appear to be somewhat more labile than those in most other tissues—presumably because post-mortal changes, especially the lactic acid formation, are more rapid in cardiac and skeletal muscle than in other tissues.

Tissue suspensions. The tissue was minced in a Latapie mill, weighed and suspended evenly in 6·5 vol. of ice-cooled saline. The saline medium was calcium-free phosphate saline (Krebs & Eggleston, 1940). Three volumes of this suspension were measured into the experimental vessel and 1 vol. of further solutions was added—substrates, usually in 0·2 M solution and/or enough saline medium to bring the concentration of the tissue in the final suspension to 10% (w/v, calc. for fresh weight). For the calculation of Q values the dry weight was assumed to be one-fifth of the wet weight. When anaerobic conditions were required, the vessel was filled with N_2 and a stick of yellow phosphorus was placed in a special compartment of the vessel to remove traces of O_2. The suspension was shaken at 40° for specified periods. At the end of this period, ½ vol. 2 N-HCl was added to stop metabolic processes. The suspension was then cooled in an ice-bath and stored in a refrigerator until it was required.

Vessels

Some experiments were carried out in conical Warburg flasks of about 20 ml. size, provided with a side-arm and a centre well. These were used when a small number of metabolites was to be analyzed, or when the O_2 uptake and the CO_2 output were to be measured. When larger amounts of tissue suspension were required for analytical purposes, either large conical manometer vessels of the dimensions shown in Fig. 1 were used, or flasks shown in Fig. 2, which resemble those described by Krebs (1932), with the addition of a centre well (to hold a stick of yellow phosphorus in anaerobic experiments) and the provision of a side-arm (from which substrates can be added after the complete elimination of O_2 by the phosphorus). These flasks were shaken in a water-bath at 40° as described by Krebs (1932).

Substrates

Oxaloacetic acid was prepared according to Wohl & Oesterlin (1901) and Wohl & Claussner (1907). It was dissolved and neutralized with M-$NaHCO_3$ immediately before use.

Preparation of acetoacetate. 2·6 ml. of recently distilled ethylacetoacetate and 10·2 ml. 2 N-NaOH were made up to 20 ml. with water and kept for 1 hr. at 40°. The solution was then neutralized with N-HCl (using litmus paper), washed into a distilling flask with 20 ml. water and reduced *in vacuo* to about 5 ml. in order to remove ethanol. The solution was transferred to a measuring flask and made up to 20 ml. This stock solution (about M) was stored in the refrigerator. Before use it was usually diluted to about 0·2 M. The exact strength of the solution used was remeasured in each experiment. The complete removal of ethanol was essential as it interferes with the determination of β-hydroxybutyric acid.

Spontaneous decomposition of acetoacetate. The occurrence of a non-enzymic 'ketone decomposition' of acetoacetate makes it difficult to estimate accurately the rate of acetoacetate metabolism when the rate of the latter is low. The spontaneous decomposition depends, among other factors, on the acetoacetate concentration and on the presence of amino compounds (Pollak, 1907; Ljunggren, 1925) and varies therefore with the conditions of the experiment.

We tried to measure the spontaneous decomposition by separating it from the metabolic decomposition by heat inactivation. The tissue suspension was warmed for 60 min. to 60°; this treatment suppresses the metabolic processes and any disappearance of acetoacetate from such an 'inactivated' suspension may therefore be attributed to non-enzymic reactions. According to Table 1 the loss of acetoacetate in sheep heart suspension of the type used in our experiments was 4–5% after 40 min. and 6·4% after 80 min. incubation. The losses recorded in the table included that incurred during the equilibration period of the manometric estimation—between 1 and 2%—and this circumstance may explain why the loss is not directly proportional

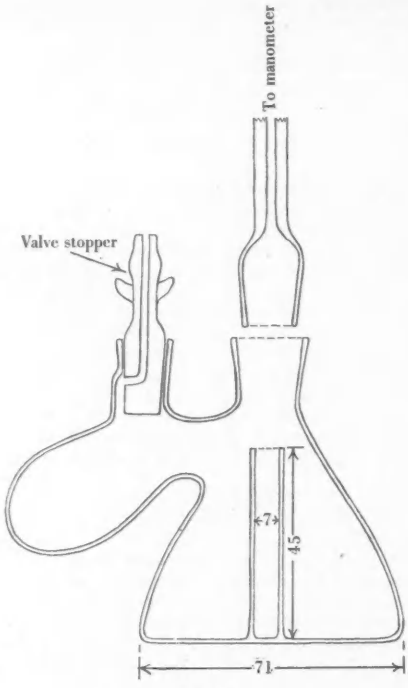

Fig. 1.

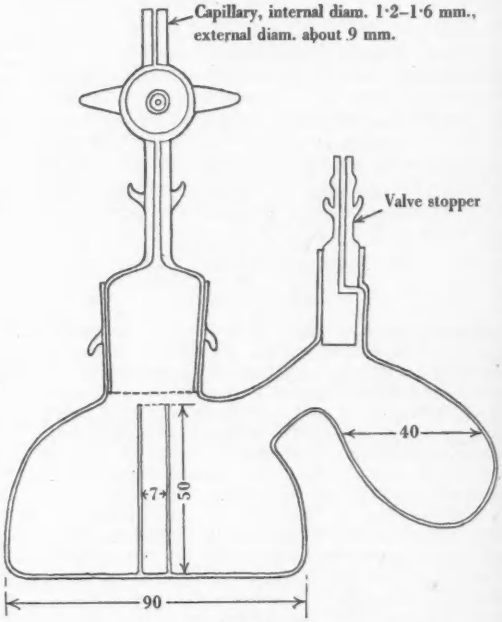

Fig. 2.

Fig. 1. Manometric flasks for large-scale experiments, holding up to 25 ml. fluid in aerobic and 60 ml. in anaerobic experiments. The side-arm holds at least 10 ml. fluid without danger of spilling when shaken in the water-bath. Total volume 110–120 ml. Dimensions in mm. $\frac{1}{4}$ actual size.

Fig. 2. Flask for metabolic experiments, with side-arm, centre tube (for NaOH or yellow phosphorus) and gas in- and outlets. The side-arm holds at least 12 ml. fluid without danger of spilling when shaken. Total volume about 250 ml. Dimensions in mm. $\frac{1}{4}$ actual size.

Table 1. *Recovery of acetoacetate added to metabolically inactive tissue*

(Tissue minced, suspended in 9 parts phosphate saline and inactivated by heat; duplicate determinations; the data refer to 4 ml. suspension; 40°.)

		Acetoacetate			
Tissue	Period of incubation (min.)	Added (μl.)	Recovered (μl.)	Loss (μl.)	Mean loss (% of that added)
Sheep heart	40	1760	1665	− 95	5·1
	40	1760	1675	− 85	
	40	2265	2185	− 80	4·0
	40	2265	2165	− 100	
	80	2265	2110	− 155	6·4
	80	2265	2130	− 135	
Sheep kidney	40	1760	1625	− 135	7·4
	40	1760	1635	− 125	

to the duration of the incubation. In kidney suspensions the loss was somewhat greater than in heart suspensions.

Throughout this paper the data on the disappearance of acetoacetate represent the actual measurements, no correction for the non-enzymic loss having been made, as this loss is usually small; but attention is drawn to the loss where it is of significance in the interpretation of the results.

Analytical methods

Acetoacetic and oxaloacetic acids. The separate manometric determination of these two β-ketonic acids, when present together, is based on the fact that aniline decarboxylates both acids, whilst Al^{+++} salts decarboxylate oxaloacetic acid only (Krebs, 1942). Both determinations were carried out directly on the supernatant of the centrifuged tissue suspension.

The aniline method for the determination of β-ketonic acids (Ostern, 1933; Edson, 1935) was modified in order to reduce the 'spontaneous' decomposition of the β-ketonic acids during the period of equilibration. The rate of the spontaneous decomposition is lowered by mineral acids,

the pH optimum being near the neutral point (Ljunggren, 1925). We therefore add enough 2N-HCl to the solution to bring its final concentration to about 0·2N. The main compartment of the vessels was filled with 3 ml. of the unknown solution (containing up to 400μl. β-ketonic acid), 0·3 ml. 2N-HCl and 0·5 ml. 50% citric acid, the side-arm with 1 ml. aniline citrate (4·5 ml. aniline plus 5·5 ml. 50% citric acid). The temperature of the bath was 25°. On mixing the reagents in the absence of β-ketonic acids a negative pressure of 14 mm. Brodie fluid was observed on the manometers. Allowance for this 'blank' was made in all measurements.

Oxaloacetic acid was determined in conical Warburg flasks provided with two side-arms, each capable of holding at least 1 ml. fluid. The main compartment contained 2 ml. of the solution to be tested, acidified with 2N-HCl to 0·2N. Side-arm one contained 1 ml. 0·75M-phthalate buffer (15·3 g. potassium hydrogen phthalate and 1·8 g. NaOH in 100 ml.), side-arm two 1 ml. 33·3% $Al_2(SO_4)_3.18H_2O$. The bath temperature was 25°. After equilibration the contents, first of side-arm one, and then of side-arm two, were mixed with the solution in the main compartment. A control experiment, measuring the changes of pressure due to mixing the various solutions and containing water instead of the unknown solution, was run parallel with the estimations. The CO_2 evolution was usually complete within 60 min. The yields were a few per cent below the theoretical amounts, probably owing to the spontaneous decomposition of oxaloacetic acid during the period of equilibration. For example, 215 and 107·5μl. were recovered from pure aqueous solutions containing 224 and 112μl. acid. In another recovery experiment, oxaloacetic acid was added to muscle extract containing 0·2N-HCl and the period of equilibration was extended to 1 hr. (usually 20 min. are required); the yields were in this case between 87·5 and 89%. The amounts of acetoacetic acid were calculated by deducting the amounts of oxaloacetic acid from those of total β-ketonic acids as determined by the aniline method.

Lactic acid was determined according to Friedemann & Graeser (1933). A suitable quantity of tissue suspension containing preferably about 2 mg. lactic acid was diluted to 40 ml. and protein and other interfering substances were removed by the copper-lime precipitation. Controls showed that malic, fumaric, citric, pyruvic, oxaloacetic and β-hydroxybutyric acids, in the quantities occurring in our samples, did not significantly interfere. Acetoacetic acid and acetone were removed by boiling the solution gently in the distilling flasks for 10 min. before $KMnO_4$ was added and before the distillate was collected.

Pyruvic acid was determined by the carboxylase method. 1 ml. of the acid (0·4N) tissue suspension, 0·4 ml. 3M-sodium acetate—to adjust the pH to 5·0—and 2 ml. water were measured into the main compartment of a conical manometer flask, and 1 ml. freshly prepared yeast extract (see Krebs & Johnson, 1937) was placed in the side-arm. If oxaloacetic acid was present the result was corrected by assuming that 1 molecule of oxaloacetic acid, when added to carboxylase, yielded 1·0 mol. CO_2 (this being the average of eight determinations). In most determinations a rapid CO_2 evolution from the decarboxylation of pyruvic and oxaloacetic acids was followed by a slow and steady evolution of CO_2, probably from α-ketoglutaric acid. The end-point of the rapid decarboxylation was ascertained by extrapolation.

Succinic acid was extracted with ether after deproteinization with tungstic acid and measured manometrically (Krebs, 1937). Usually 3–5 ml. suspension were used. The ether for the extraction must be free from aldehyde (2 ml. Nessler's reagent must remain clear when shaken with 5 ml. ether), the presence of which causes loss of succinic acid. Small amounts of acetaldehyde were removed by shaking the ether with alkaline $KMnO_4$ (saturated solution of $KMnO_4$ in N-NaOH).

α-Ketoglutaric and α-hydroxyglutaric acids. As cardiac muscle reduces α-ketoglutaric acid to α-hydroxyglutaric acid (Weil-Malherbe, 1937), the two acids are likely to occur together. The sum of the acids was measured as follows (see Krebs, 1938). A portion of the protein-free filtrate (deproteinized with tungstic acid) corresponding to about 4 or 5 ml. tissue suspension, was diluted to about 15 ml. and acidified with 2 ml. 50% H_2SO_4. Then first 5 ml. 4·7% $KMnO_4$ and afterwards 0·5 ml. acid $MnSO_4$ were added and allowed to react for 60 min. at room temperature. The excess of oxidizing agent was removed by addition of enough acid $FeSO_4$ solution (200 g. $FeSO_4.7H_2O$ and 10 g. H_2SO_4 in 1 l.) to turn the colour of the solution pale green. The succinic acid formed was extracted and determined. When succinate was present in the original suspension, this was determined in a separate portion. The difference between succinic acid found after direct extraction and succinic acid found after treatment with $KMnO_4$ represents the sum of α-keto- and α-hydroxyglutarate. Glutamic acid does not react in significant quantities under the above conditions. Using this procedure we recovered about 95% α-ketoglutarate and about 90% α-hydroxyglutarate from pure solutions.

For the separate determination of α-ketoglutaric and α-hydroxyglutaric acids an excess of 2:4-dinitrophenylhydrazine (1% solution in 10% H_2SO_4) was added to the deproteinized filtrate. After standing 30 min., the hydrazones were extracted with aldehyde-free ether and oxidized to succinic acid as previously described (Krebs, 1938). Before the extraction of succinic acid, $FeSO_4$ solution was added as stated above. The aqueous phase containing α-hydroxyglutaric and succinic acids was mixed with 1 ml. acetone to remove the excess dinitrophenylhydrazine. The volume was measured and the precipitate filtered off after 30 min. A known quantity of the filtrate was boiled to remove the excess of acetone. After cooling, the solution was acidified, treated with $KMnO_4$ and extracted as stated for the determination of the sum of the keto- and hydroxy-acids. Recovery from pure mixtures of the two acids was about 95% for α-ketoglutaric acid and 75% for α-hydroxyglutaric acid.

Fumaric and malic acids. Fumaric acid was estimated by the method of Krebs, Smyth & Evans (1940), by reduction to succinic acid and manometric determination of the latter using 3–5 ml. suspension. In previous work from this laboratory the concentration of *l*(−)-malic acid was calculated from the equilibration constant on the assumption that owing to the powerful activity of fumarase the two acids are virtually present as the equilibrium mixture. This assumption was based on the observation that under experimental conditions the equilibrium is established within a few minutes, 5–10 min. when fumarate was added (see Table 2), and even more rapidly when *l*(−)-malate was added. In these experiments, however, *sec.*-octanol had been added to the suspensions in order to eliminate oxi-

Table 2. *Rate of conversion of fumarate into l(−)-malate in sheep heart*

(6 g. (fresh weight) minced sheep heart (frozen and stored for 6 days), 48 ml. phosphate saline; 6 ml. 0·1 M-fumarate, one drop octanol, incubated at 40°. *l*(−)-Malate estimated polarimetrically at intervals in 8 ml. samples.)

Period of incubation (min.)	$[\alpha]_D$ observed (°)	*l*(−)-Malic acid formed (mg./ml. solution)
2·5	0·95	0·77
5	1·12	0·90
10	1·18	0·95
20	1·19	0·96*

* On the assumption that the ratio *l*(−)-malate/fumarate, in the equilibrium mixture is 3·17 (Krebs, Smyth & Evans, 1940), 1·02 mg. *l*(−)-malic acid are expected.

dative enzymes. In the course of the present work we found that the proportion of fumaric to malic acids in the metabolically active tissue differed frequently from the expected ratio. The ratio malate/fumarate was usually much lower than it is in tissue from which the energy-giving reactions have been eliminated. For example, when a suspension of fresh sheep heart in 9 parts of phosphate saline was shaken for 30 min. with fumarate (final concentration 0·02 M) the following ratios were observed (40°).

Shaken in O_2: ratio malate/fumarate, 1·75; 1·56 (duplicates).

Shaken in N_2: ratio malate/fumarate, 1·53; 1·65 (duplicates).

When the same cardiac muscle was used after washing with water and addition of octanol, the ratio measured under otherwise identical conditions agreed within the limits of error with the previously reported figure of 3·17. It was thus not permissible to calculate the concentration of malic acid from the equilibrium constant. We used the polarimetric method of Auerbach & Krüger (1923), as modified by Krebs & Eggleston (1943), for the independent determination of malic acid; 8 ml. unknown solution which contained 0·2 N-HCl, 2 ml. M-trisodium citrate, 1 ml. glacial acetic acid, 29% (w/v) aqueous commercial ammonium molybdate, freshly prepared solution, and about 0·5 g. charcoal, were mixed and filtered and the rotation was read in 2 dm. tubes. Under the conditions employed $[\alpha]_D^{20°}$ was +1550° and a reading of 1° indicated the presence of 0·806 mg. malic acid/ml. of the unknown solution under the same conditions. $[\alpha]_D^{20°}$ of the molybdate complexes of *l*(+)-lactic acid was −84°; of *l*(−)-α-hydroxyglutaric acid −76°; of (−)-*iso*citric acid −780°. The quantities of these three acids in our solutions were usually so small that their effect on the rotation could be neglected. In some cases correction factors based on the above figures were used.

As for the explanation of the differences of the ratios malate/fumarate in fresh and treated tissue, the assumption that the differences are due to the circumstance that the two reactions establishing the equilibrium are outpaced by other reactions of fumarate and malate upsetting the equilibrium is not supported by the available evidence; fumarase seems to be fully active in fresh tissue; the potential rates of the reactions catalyzed by this enzyme, therefore, are at least 10 times greater than the rates of other reactions of fumarate and malate. It is more probable that the environment of the enzyme is different in fresh and treated tissue. The enzyme may be localized in a lipid phase where the relative concentrations of the two acids and of water are different. Jacobsohn (1934), Jacobsohn, Pereira & Tapadinhas (1932) and Jacobsohn & Tapadinhas (1934) also noted that the equilibrium depends on the source and treatment of the enzyme preparation.

β-Hydroxybutyric acid. A modification of Van Slyke's mercuric sulphate method was used (see Edson, 1935). A quantity of the solution containing preferably between 1 and 2 mg. of the acid was diluted to 40 ml. in a stoppered 50 ml. measuring cylinder and mixed with 5 ml. 20% (w/v) $CuSO_4.5H_2O$ and 5 ml. 20% (w/v) $Ca(OH)_2$ suspension. After 30 min. the suspension was filtered and 30 ml. filtrate were transferred to a 250 ml. ground-glass boiling flask; 2·4 ml. 50% (v/v) H_2SO_4 and 8·4 ml. 10% (w/v) $HgSO_4$ were then added. The solutions were boiled under reflux for 30 min. and then allowed to stand overnight. The precipitate, containing the mercuric complexes of acetone, pyruvic acid, and possibly of other substances, were separated from the solution by suction through a Jena G4 sintered glass funnel and washed with a few ml. 5% sulphuric acid. The further treatment was as described by Edson (1935) except that an extra amount—0·5 ml.—of 7·5 N-NaOH was added to facilitate the reduction of the Hg ions, and 10 min. were allowed for the reduction. After addition of iodine, the solution was allowed to stand until it was completely clear (30–60 min.). Recovery of β-hydroxybutyrate added to inactivated tissue suspensions was quantitative.

This method, like all other micro-methods for the determination of β-hydroxybutyric acid, suffers from lack of specificity. However, we have convinced ourselves that interfering substances, among them lactic acid and ethanol, did not occur in significant quantities in our material, for β-hydroxybutyric acid was found only when acetoacetic acid had been added. In control suspensions similar quantities of substances which might be expected to interfere—citric, malic, fumaric, α-ketoglutaric, lactic acids—yielded no measurable amount of 'β-hydroxybutyric acid'.

Citric, l(−)-iso*citric and* cis-*aconitic acids* were determined according to the methods described by Krebs & Eggleston (1944). The colorimetric procedure was followed whenever β-hydroxybutyric acid was present or likely to be present. The three tricarboxylic acids were always found to be present as their equilibrium mixture.

Total CO_2 was measured manometrically according to Warburg & Yabusoe (1924).

RESULTS

Aerobic and anaerobic removal of acetoacetate. Data showing the order of magnitude of the rate of acetoacetate removal in minced sheep heart are shown in Table 3. The data all refer to suspensions where acetoacetate was the only substrate added. It will be seen that under these conditions two to three times more acetoacetate is removed aerobically than anaerobically.

Effect of malonate. Jowett & Quastel (1935), working with kidney tissue, found that the aerobic removal of acetoacetate is inhibited by malonate

Table 3. *Removal of acetoacetate under aerobic and anaerobic conditions in sheep heart*

(The data refer to 4 ml. suspension.)

Exp. no.	Initial amount of aceto-acetate (μl.)	Period of incu-bation (min.)	Final amount of acetoacetate (μl.) Aerobic	Anae-robic	$Q^{O_2}_{ac.-ac.}$	$Q^{N_2}_{ac.-ac.}$
1	1776	30	1073	1535	−17·6	−6·0
2	1752	30	1233	—	−13·0	—
3	1752	30	1220	1490	−13·3	−6·6
4	908	40	—	679	—	−4·3
5	888	40	—	604	—	−5·3
6	1866	30	—	1546	—	−8·0
7	1814	30	—	1432	—	−9·6
8	1732	40	1145	1375	−11·4	−7·1

whilst the anaerobic removal is not affected. This is also true for minced sheep heart, as will be seen from Table 4: even 0·001 M-malonate has a powerful effect. Malonate reduces the rate of the aerobic to that of the anaerobic removal.

Table 4. *Effect of malonate on the removal of acetoacetate in sheep heart*

(0·02 M-acetoacetate; 40 min. incubation; the data refer to 4 ml. suspension.)

Final concentration of substances added	Gas	Acetoacetate removed from 4 ml. medium (μl.)	$Q_{ac.-ac.}$
—	O_2	703	−17·6
0·001 M-malonate	O_2	258	− 6·5
0·01 M-malonate	O_2	166	− 4·2
—	N_2	241	− 6·0
0·01 M-malonate	N_2	286	− 7·2

Inhibitions by low concentrations of malonate suggest—if not prove—that succinic dehydrogenase plays a part in the removal of acetoacetate. This assumption served as the working hypothesis for the further experiments.

Acceleration of acetoacetate removal. It seemed probable, bearing in mind the relations between succinic dehydrogenase and pyruvate metabolism in muscle tissue (Krebs & Eggleston, 1940), that the removal of acetoacetate depends on substances formed from succinate; the effect of the various acids of the tricarboxylic acid cycle was therefore examined. Since the substances are interconvertible under aerobic conditions, no clear-cut results can be expected in the presence of O_2 and anaerobic conditions were therefore chosen. These do not completely exclude interconversion of some of the members of the cycle, especially in the presence of an excess of oxaloacetate, but they reduce the possibilities of interconversion.

Four of the eight acids of the cycle—succinate, citrate, *iso*citrate, *cis*-aconitate—had no effect on the anaerobic removal of acetoacetate; the other four—fumarate, *l*(−)-malate, oxaloacetate and α-ketoglutarate—greatly increased the rate (Tables 5 and 6) and raised it to, or even above, the level of the aerobic removal.

The increase was also observed under aerobic conditions, but the effect was less marked. When malonate is present, however, the four acids increased the aerobic acetoacetate removal almost as much as the anaerobic removal. They almost abolished the malonate inhibition (Table 6). These results support the view that metabolites arising in the tissue through the intermediation of succinic

Table 5. *Acceleration of the anaerobic removal of acetoacetate in sheep heart*

(The data refer to 4 ml. suspension.)

Exp. no.	Gas	Initial concentration of aceto-acetate (M)	Substance added	Final concentration (M)	Period of incubation (min.)	Aceto-acetate removed (μl.)	$Q_{ac.-ac.}$
1	N_2	0·01	—	—	20	193	− 7·2
			Fumarate	0·01		494	−18·5
			l(−)-Malate	0·01		450	−16·9
			Oxaloacetate	0·01		456	−17·1
			α-Ketoglutarate	0·01		334	−12·5
2	N_2	0·02	—	—	30	241	− 6·0
			Fumarate	0·005		768	−19·2
3	N_2	0·02	—	—	30	262	− 6·6
			Oxaloacetate	0·02		667	−16·7
			α-Ketoglutarate	0·02		717	−17·9
4	N_2	0·02	—	—	40	377	− 7·1
			Oxaloacetate	0·01		1062	−19·9
			Fumarate	0·01		1339	−25·1
			α-Ketoglutarate	0·01		819	−15·3
5	O_2	0·02	—	—	40	607	−11·4
			Fumarate	0·01		1092	−20·5
			Oxaloacetate	0·01		1114	−20·9
			α-Ketoglutarate	0·01		877	−16·4

dehydrogenase play a part in the removal of acetoacetate.

Table 6. *Effect of fumarate, oxaloacetate and α-ketoglutarate on the removal of acetoacetate by sheep heart, in the absence and presence of malonate*

(0·02 M-acetoacetate; 60 min. incubation; the data refer to 4 ml. suspension.)

	Further substrates added			Acetoacetate removed (μl.)	
Exp. no.	Substance	Final concentration (M)	Gas	No malonate	With 0·01 M-malonate
1	None	—	O_2	950	120
	Fumarate	0·02	O_2	1212	924
	α-Ketoglutarate fumarate	0·02	O_2	1243	545
	α-Ketoglutarate	0·02	O_2	1472	1070
2	None	—	N_2	420	330
	α-Ketoglutarate	0·02	N_2	900	735
	Fumarate	0·02	N_2	1395	1192
	Oxaloacetate	0·02	N_2	1190	1185
	l(−)-Malate	0·02	N_2	1612	1240
3	None	—	O_2	870	148
	α-Ketoglutarate	0·02	O_2	838	363
	Fumarate	0·02	O_2	1068	858
	Oxaloacetate	0·02	O_2	805	768
	l(−)-Malate	0·02	O_2	1055	623

Quantitative aspects of the effects of fumarate, oxaloacetate and α-ketoglutarate. In the previous experiments acetoacetate and the second substrate were added in approximately molecular proportions. In Tables 7 and 8 experiments are recorded in which the amount of acetoacetate was considerably in excess of the amounts of fumarate or α-ketoglutarate. It will be seen that α-ketoglutarate (Table 7) causes the removal of an equivalent quantity of acetoacetate, while in the case of fumarate and oxaloacetate (Table 8) no simple molecular proportion emerges. One molecule of oxaloacetate, for example, causes the disappearance of up to 4 molecules of acetoacetate. The effects of fumarate and oxaloacetate thus are catalytic.

Table 7. *Effect of α-ketoglutarate on the anaerobic removal of acetoacetate in sheep heart*

(Initial concentration of acetoacetate 0·02 M; N_2; 60 min. incubation. The data refer to 4 ml. at 40°.)

α-Ketoglutarate added (μl.)	0	112	224	448	896
Acetoacetate removed (μl.)	300	420	515	750	870
Extra acetoacetate removed on addition of α-ketoglutarate (μl.)	—	120	215	450	570

Table 8. *Effect of fumarate and oxaloacetate on the anaerobic removal of acetoacetate in sheep heart*

(Initial concentration of acetoacetate 0·036 M; N_2; incubation 120 min.; the data refer to 4 ml. suspension.)

		Fumarate		Oxaloacetate	
Further substrate added (μl.)	None	179	89·5	179	89·5
Acetoacetate removed (μl.)	220	585	470	860	565
Extra acetoacetate removed owing to addition of fumarate or oxaloacetate (μl.)	—	365	250	640	345

Mechanism of action of α-ketoglutarate. The results of two experiments in which approximately equimolecular quantities of acetoacetate and α-ketoglutarate were added anaerobically to minced sheep heart and the changes in the concentrations of relevant metabolites were measured are shown in Table 9. When α-ketoglutarate alone was added, part of it disappeared and equivalent amounts of α-hydroxyglutarate and succinate were formed, in

Table 9. *Acetoacetate and α-ketoglutarate in sheep heart*

Vessel no. ...	(1)	(2)	(3)
Exp. 1. 48 ml. suspension incubated for 30 min. anaerobically			
Metabolites added (μl.):			
Acetoacetate	10,900	10,900	Nil
α-Ketoglutarate	Nil	10,750	10,750
Metabolites found (μl.):			
Acetoacetate	8,620	6,100	Nil
β-Hydroxybutyrate	1,450	3,915	Nil
α-Hydroxy- plus α-ketoglutarate	335	6,810	10,200
Succinate	110	3,990	285
CO_2 plus HCO_3 (formed)	1,800	5,550	1,800
Changes in metabolites (μl.):			
Acetoacetate	−2,280	−4,800	Nil
β-Hydroxybutyrate	+1,450	+3,915	Nil
α-Hydroxy- plus α-ketoglutarate	+ 335	−3,940	− 550
Succinate	+ 110	+3,990	+ 285
CO_2 plus HCO_3	+1,800	+5,500	+1,800
Exp. 2. 48 ml. suspension incubated for 60 min. anaerobically			
Metabolites added (μl.):			
Acetoacetate	Nil	21,990	Nil
α-Ketoglutarate	21,500	21,500	Nil
Metabolites found (μl.):			
Acetoacetate	Nil	13,230	Nil
Succinate	2,005	5,540	415
α-Ketoglutarate	17,350	12,600	Nil
α-Hydroxyglutarate	2,220	2,990	1,005
Changes in metabolites (μl.):			
Acetoacetate	Nil	−8,760	Nil
Succinate	+2,005	+5,540	+ 415
α-Ketoglutarate	−4,150	−8,900	Nil
α-Hydroxyglutarate	+2,220	+2,990	+1,005

accordance with the dismutation described by Weil-Malherbe (1937),

$$2\ \alpha\text{-ketoglutarate} + H_2O = \alpha\text{-hydroxyglutarate} + \text{succinate} + CO_2. \quad (3)$$

When both acetoacetate and α-ketoglutarate were present, a greater amount of α-ketoglutarate disappeared, the excess being approximately equivalent to the extra removal of acetoacetate. At the same time the yield of succinate increased. Acetoacetate was largely recovered as β-hydroxybutyrate. Such acetoacetate as was not recovered could be accounted for by the non-enzymic ketone decomposition. The interaction between acetoacetate and α-ketoglutarate may thus be attributed to the reaction already found by Krebs & Johnson (1937) to occur in kidney and brain:

$$\alpha\text{-ketoglutarate} + \text{acetoacetate} + H_2O = \text{succinate} + CO_2 + \beta\text{-hydroxybutyrate}. \quad (4)$$

Acetoacetate and fumarate. The results of an experiment in which acetoacetate and fumarate were added anaerobically to sheep heart are recorded in Table 10. It will be seen that in vessel 3 the centration during the incubation was therefore much lower than in vessel 1 and the deficit, if any, is therefore expected to be greater in 2 than in 3.

Of the added fumarate 72 % was recovered either as fumarate or as malate in the control vessel 1 (where no other substrate was added). The rest was converted into succinate, α-keto- and α-hydroxyglutarate. This may be explained by a series of oxido-reductions, the primary reaction being:

$$\text{fumarate} + \text{malate} = \text{succinate} + \text{oxaloacetate}.$$

This reaction was observed by Green (1936) and Dewan & Green (1937) in certain enzyme preparations. It would be followed by the series of reactions of oxaloacetate reported by Krebs, Eggleston, Kleinzeller & Smyth (1940) in which part of the oxaloacetate is reduced to malate whilst part enters oxidative reactions yielding the tricarboxylic acids and α-ketoglutarate. The latter then undergoes dismutation (3).

The effect of fumarate on the removal of acetoacetate (vessel 3) is, as in previous experiments,

Table 10. *Acetoacetate and fumarate in sheep heart*

(60 ml. suspension incubated for 90 min. anaerobically (substrate concentration about 0·02 M).)

Vessel no. ...	(1)	(2)	(3)	(4)
Metabolites added (μl.):				
Acetoacetate	Nil	27,420	27,420	Nil
Fumarate	26,880	Nil	26,880	Nil
Metabolites found (μl.):				
Acetoacetate	—	19,800	1,275	Nil
β-Hydroxybutyrate	—	4,410	28,225	Nil
Succinate	3,740	360	5,810	330
α-Ketoglutarate	760	48	1,080	260
α-Hydroxyglutarate	2,100	1,445	2,860	1,170
Fumarate	5,660	Nil	5,330	210
l(−)-Malate	13,660	Nil	10,820	505
Citrate	Nil	Nil	750	Nil
Oxaloacetate	Nil	Nil	Nil	Nil
Pyruvate	Nil	Nil	Nil	Nil
Changes in metabolites (μl.):				
Acetoacetate	—	−7,620	−26,145	—
β-Hydroxybutyrate	—	+4,410	+28,225	—
Succinate	+3,740	+ 360	+ 5,810	—
α-Ketoglutarate	+ 760	+ 48	+ 1,080	—
α-Hydroxyglutarate	+2,100	+1,445	+ 2,860	—
Fumarate + *l*(−)-malate	−7,560	Nil	−10,730	—
Citrate	—	—	+ 750	—

acetoacetate removed agrees within the limits of error with the β-hydroxybutyrate recovered. In the control vessel 2, the yield of β-hydroxybutyrate is somewhat low, but the deficit is of the order expected on account of the ketone decomposition of acetoacetate. The loss by this side reaction is, of course, the greater the lower the rate of acetoacetate metabolism. In vessel 3, acetoacetate was almost completely removed. The average acetoacetate concatalytic; the extra amount of acetoacetate removed as a result of the addition of fumarate (difference between vessels 2 and 3) is 18,500 μl. The extra amount of fumarate removed as a result of the presence of acetoacetate (difference between fumarate + malate in vessels 1 and 3) is 3170 μl. Thus 1 molecule of fumarate has caused the disappearance of at least 5·8 molecules of acetoacetate. The effect of fumarate on the removal of acetoacetate can be

satisfactorily explained if it is assumed that the following primary reaction takes place:

malate + acetoacetate
= oxaloacetate + β-hydroxybutyrate (5)

Oxaloacetate formed by this reaction is expected to disappear almost at once (see Krebs, Eggleston, Kleinzeller & Smyth, 1940). It is not possible to formulate exactly the coupled oxido-reductions taking place after addition of fumarate and acetoacetate, as several combinations of the following reactions are feasible:

Reductions:

acetoacetate → β-hydroxybutyrate
α-ketoglutarate → α-hydroxyglutarate
fumarate → succinate

Oxidations:

malate → oxaloacetate
triose or triosephosphate → pyruvic acid
oxaloacetate + pyruvate → tricarboxylic acids (citric, *iso*citric and *cis*-aconitic acids)
tricarboxylic acids → α-ketoglutarate
α-ketoglutarate → succinate

Succinate, it will be noted, can be formed in two different ways. How much is formed by reduction of fumarate and how much by oxidation of α-ketoglutarate can be approximately estimated from the oxidation reduction balance of the system. The calculation is not completely accurate because certain small fractions of some metabolites were not formed during the incubation but were present from the start, as the figures referring to the control vessel 4 indicate. The preformed quantities were not determined in this experiment; being small they were neglected in the calculation of Table 11. The table shows that oxidations and reductions balance if it is assumed that half of the succinate is formed by reduction and half by oxidation. Another balance sheet comparing the number of fumarate molecules removed with the number of molecules formed from fumarate gives an indication of how much of the pyruvate required for the formation of the tricarboxylic acids (and their oxidation products α-ketoglutarate and succinate) is derived from cell material (carbohydrate) and how much from the ketone decomposition of oxaloacetate. If pyruvate comes from carbohydrate, one molecule of fumarate is needed for each molecule of tricarboxylic acid, or α-ketoglutarate, or succinate. If pyruvate is derived from oxaloacetate, and thus ultimately from the added fumarate, two molecules of fumarate are needed for each molecule of tricarboxylic acid, α-ketoglutarate and (oxidatively formed) succinate. The data given in Table 12 indicate that, in all, 6000 μl. of pyruvate were required for the formation of citrate, α-keto- and α-hydroxyglutarate and succinate. About one-third of this (10,730 − 8740 = 1990 μl.) could have been derived from fumarate. The rest must have come from other sources, probably carbohydrate. Determinations of lactate in the suspensions showed no appreciable difference between controls and those containing fumarate and acetoacetate.

The oxidative formation of citrate, α-keto- and α-hydroxyglutarate and succinate involves a liberation of CO_2. The CO_2 output was measured in a separate experiment recorded in Table 13. These measurements were not very accurate because ketone decomposition of acetoacetate was fairly rapid under the conditions of CO_2 determination. The figures for total CO_2 in Table 13 have been corrected for this decomposition, but are probably

Table 11. *Oxido-reduction balance of the reactions taking place in sheep heart after addition of fumarate and acetoacetate*

(Based on data for vessel 3 of Table 10. The figures refer to O- (or 2 H-) equivalents, the reductive formation of β-hydroxybutyrate, of β-hydroxyglutarate and of succinate being taken to require 1 O-equivalent, the oxidative formation of citrate being taken to require 3 O-equivalents, of α-keto- and α-hydroxyglutarate 4 O-equivalents, of succinate 5 O-equivalents.)

	O-equivalents (μl.)	Total
Reductions:		
Formation of β-hydroxybutyrate	28,225	
Formation of α-hydroxyglutarate	2,860	33,990
Formation of succinate (half of total)	2,905	
Oxidations:		
Formation of citrate	2,250	
Formation of α-ketoglutarate	4,320	
Formation of α-hydroxyglutarate	11,440	32,540
Formation of succinate (half of total)	14,530	

Table 12. *Fumarate used and products of fumarate found in the presence of acetoacetate*

(Based on data for vessel 3 of Table 10.)

	μl.	Total
Equivalents of fumarate removed (not counting those converted into malate)	10,730	—
Equivalents recovered:*		
1. Assuming that 1 mol. fumarate gives 1 mol. end-product:		
Citrate	750	
α-Keto- and α-hydroxyglutarate	2,510	8,740
Succinate	5,480	
2. Assuming that citrate, α-keto- and α-hydroxyglutarate and one half of the succinate require 2 mol. of fumarate, and the other half of succinate 1 mol.:		
Citrate	1,500	
α-Keto- and α-hydroxyglutarate	5,020	14,740
Succinate	8,220	

* Corrected for quantities preformed in the tissue according to vessel 4 (Table 10).

still a little too high. The total CO_2 formation was about 80% of the β-hydroxybutyrate found, which is of the expected order of magnitude.

Table 13. *Liberation of* CO_2 *after addition of fumarate and acetoacetate to sheep heart*

(The data refer to 4 ml. suspension incubated anaerobically for 60 min.)

Substrates added (0·02 M final concentration)	Fumarate	Acetoacetate	Fumarate plus acetoacetate
CO_2 plus HCO_3' formed (μl.)	+20	+118	+1017
Acetoacetate removed (μl.)	—	−175	−1153
β-Hydroxybutyrate formed (μl.)	Nil	+219	+1260

Acetoacetate and oxaloacetate. Fumarate in the foregoing experiments can be replaced by *l*(−)-malate without significant effect on the results.

Many experiments were carried out where oxaloacetate replaced fumarate. An example is given in Table 14. The results are in agreement with the view that oxaloacetate does not react as such with acetoacetate, but only after reduction to malate. Oxaloacetate alone forms malate, fumarate and some succinate by reduction, and citrate, α-ketoglutarate, α-hydroxyglutarate and some succinate by oxidation. When acetoacetate is present together with oxaloacetate, the former is reduced to β-hydroxybutyrate and the quantities of malate and fumarate formed are smaller, whilst those of the oxidation products (citrate, α-keto- and α-hydroxyglutarate, and succinate) are increased.

The following two points deserve special mention. (1) The acetoacetate removed is completely accounted for by the β-hydroxybutyrate formed. None of the acetoacetate can therefore have reacted in the manner assumed by Breusch (1943) and Wieland & Rosenthal (1943). (2) In the presence of acetoacetate some of the added oxaloacetate is recovered unchanged. It is unlikely, in view of the instability of this substance, that the recovered oxaloacetate is a remnant of the originally added compound, since no oxaloacetate is found in the absence of acetoacetate. It is more probable that oxaloacetate is found because it is continuously reformed from malate according to reaction (5).

Table 14. *Acetoacetate and oxaloacetate in sheep heart*

(60 ml. suspension, incubated for 80 min. anaerobically; except vessel 4 which was acidified at the start to give the initial quantities of metabolites in the tissue.)

Vessel no. ...	(1)	(2)	(3)	(4)
Metabolites added (μl.):				
Acetoacetate	—	49,800	49,800	—
Oxaloacetate	26,900	26,900	—	—
Metabolites found (μl.):				
Acetoacetate	Nil	19,100	45,600	Nil
Oxaloacetate	Nil	1,275	Nil	Nil
β-Hydroxybutyrate	~Nil	31,500	5,950	~Nil
Succinate	4,690	6,490	240	~Nil
l(−)-Malate	8,660	3,480	~Nil	~400
Fumarate	3,550	3,260	~540	~550
Citrate, *iso*citrate and *cis*-aconitate	850	1,320	340	360
Pyruvate	5,480	2,265	Nil	Nil
Lactate	11,220	9,800	11,480	7,300
α-Ketoglutarate	890	2,430	125	90
α-Hydroxyglutarate	1,490	3,410	590	870
Changes in metabolites (μl.):*				
Acetoacetate	—	−30,700	−4,200	—
β-Hydroxybutyrate	—	+31,500	+5,950	—
Oxaloacetate	−26,900	−25,625	—	—
Succinate	+ 4,690	+ 6,490	+ 240	—
l(−)-Malate	+ 8,260	+ 3,080	− 400	—
Fumarate	+ 3,000	+ 2,710	Nil	—
Citrate, *iso*citrate and *cis*-aconitate	+ 490	+ 960	~Nil	—
Pyruvate	+ 5,480	+ 2,265	—	—
Lactate	+ 3,920	+ 2,500	+4,180	—
α-Ketoglutarate	+ 800	+ 2,340	~Nil	—
α-Hydroxyglutarate	+ 620	+ 2,540	~Nil	—

* Corrected for preformed metabolites as found in vessel 4.

AEROBIC REACTIONS OF ACETOACETATE

The experiments reported so far deal chiefly with the mechanism of the anaerobic reactions of acetoacetate in sheep heart. In the presence of O_2 further reactions occur which will be dealt with in a subsequent paper.

ACETOACETATE IN SHEEP KIDNEY

A number of the foregoing experiments were repeated with minced sheep kidney cortex. The rates of removal of acetoacetate in this tissue were about 50% of the rates observed in cardiac muscle. The effects of malonate, fumarate, oxaloacetate and α-ketoglutarate were of the same type and of the same order of magnitude. The products of the interaction between these substances and acetoacetate were also the same.

DISCUSSION

The hypotheses of Breusch and of Wieland & Rosenthal. The observations on the anaerobic removal of acetoacetate are accounted for by the reaction (4) and (5) and do not make it necessary to postulate additional anaerobic reactions. No doubt further reactions occur aerobically, as animal tissues can oxidize ketone bodies to completion. The hypotheses of Breusch (1943) and of Wieland & Rosenthal (1943) assume the first step of the oxidative breakdown of acetoacetate to be the anaerobic reactions (1) or (2) respectively. As these reactions do not require O_2 they would be expected to occur under the conditions of our anaerobic experiments if the hypotheses are correct. In fact the reactions did not occur. As virtually all acetoacetate could be recovered either as β-hydroxybutyrate (or unchanged), the occurrence of the reactions (1) or (2) under the conditions of our experiments can be definitely excluded. Breusch and Wieland & Rosenthal did not examine the formation of β-hydroxybutyrate. Their hypotheses were merely based on the observation that acetoacetate increases the yield of citrate formed in the presence of oxaloacetate. However, this observation can be explained otherwise: when oxaloacetate alone is added to the tissue a large proportion undergoes reduction to malic acid, whilst another portion enters oxidative reactions yielding, among other substances, citrate. Acetoacetate causes the reoxidation of malate to oxaloacetate, and thus makes a greater proportion available for the reactions leading to citrate.

Oxidation-reduction potentials. According to Green, Dewan & Leloir (1937), E_0' of the system β-hydroxybutyrate ⇌ acetoacetate is −0·282 V. (38°; pH 7·0). According to Laki (1937), E_0' of the system $l(-)$-malate ⇌ oxaloacetate is −0·169 V. (37°; pH 7·0). Therefore an equilibrium should exist when

$$\frac{[\text{malate}] \times [\text{acetoacetate}]}{[\text{oxaloacetate}] \times [\beta\text{-hydroxybutyrate}]} = 6 \times 10^9.$$

The reaction malate + acetoacetate → oxaloacetate + β-hydroxybutyrate is thus expected to be stopped at a very early stage by the products of the reaction. Actually this does not happen. The fact that oxaloacetate is very rapidly removed by secondary reactions does not account for this apparent discrepancy. Its cause requires further investigation.

SUMMARY

1. Acetoacetate was added to suspensions of minced sheep heart muscle under varying conditions. The chemical changes associated with the disappearance of acetoacetate were investigated.

2. Flasks for large-scale metabolic experiments, holding between 40 and 150 ml. tissue suspension, are described.

3. Manometric methods for the separate determination of oxaloacetate and acetoacetate are described. The former is measured by decarboxylation with aluminium sulphate and the sum of β-ketonic acids by decarboxylation with aniline citrate. Modifications and special details of the methods for the determination of succinic, α-ketoglutaric, α-hydroxyglutaric, fumaric, $l(-)$-malic and β-hydroxybutyric acids are described.

4. $Q_{\text{acetoacetate}}$ was about −15 in O_2 and −6 in N_2 when the concentration of the substrate was 0·02 M and when no other substrates were added. These rates are considerably higher than those previously reported for animal tissues.

5. The aerobic removal of acetoacetate is inhibited by 0·001 M-malonate, whilst the anaerobic removal is not affected. The inhibition is taken to indicate that succinic dehydrogenase plays a part in the aerobic removal of acetoacetate.

6. Fumarate, $l(-)$-malate, oxaloacetate and α-ketoglutarate increase the rates of the aerobic and anaerobic removal of acetoacetate, the latter more than the former. In the presence of these substances the difference between the aerobic and anaerobic rates are small. The same substances largely abolish the inhibition by malonate.

7. The effect of α-ketoglutarate was found to be due to the reaction: acetoacetate + α-ketoglutarate + H_2O = β-hydroxybutyrate + succinate + CO_2.

8. The anaerobic effects of fumarate, $l(-)$-malate and oxaloacetate can be accounted for by the assumption that the reaction: acetoacetate + $l(-)$-malate = β-hydroxybutyrate + oxaloacetate takes place. Oxaloacetate does not appear to react directly with acetoacetate, but only after reduction to $l(-)$-malate.

9. Under the conditions of the anaerobic experiments virtually all metabolized acetoacetate was recovered as β-hydroxybutyrate. This is evidence against the hypotheses of Breusch (1943) and of Wieland & Rosenthal (1943), which assume direct anaerobic formation of citrate from oxaloacetate and acetoacetate. An alternative explanation for the yields of citrate observed by Breusch and by Wieland & Rosenthal is proposed. It is pointed out that the evidence presented by these authors in support of their hypotheses is inconclusive.

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The Effect of Various Chemical and Physical Agents on the Dehydrogenating Enzymes of *Eberthella typhosa*

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Knowledge of dehydrogenating enzymes has become a useful tool in the study of the nutrition of bacteria. The effect of physical and chemical agents on such dehydrogenation systems has been studied by many workers. Quastel & Wooldridge (1927*a*) found that exposure of *Escherichia coli* to various chemical and physical injuries resulted in considerable and partly selective inhibition of the dehydrogenating mechanisms. Cell destruction produced by repeated freezing and thawing has been studied by Young (1929), Yudkin (1937*b*) and Adler, Hellström, Günther & v. Euler (1938). The selective action of many types of injury forms the basis of the theory of dehydrogenating mechanisms formulated by Quastel and co-workers (Quastel, 1926; Quastel & Wooldridge, 1927*b*), which stresses the importance of the structural integrity of the cell.

The present investigation deals with the dehydrogenating mechanisms of *Eberthella typhosa* and the effect of chemical and physical conditions on these enzymes. Previous experiments on the influence of physical and chemical factors on bacterial dehydrogenases have been made on *Esch. coli*; it seemed, therefore, of interest to study these problems in connexion with a pathogenic micro-organism with different fermentative properties.

GENERAL METHODS

The following strains of *E. typhosa* were used: the motile H 901 and Vi 2, and the non-motile O 901. The bacteria were grown on ordinary nutrient agar at 37°. After 24 hr. incubation the cultures were suspended in saline and washed three times. Longer incubation than 24 hr. decreased the dehydrogenating abilities of the bacteria. For the detection of hydrogen donators the procedure previously described (Guggenheim, 1944) was used, methylene blue serving as H acceptor. In spite of carefully controlled conditions the dehydrogenase content found for individual bacterial suspensions varied. Consequently, each substrate was tested four to five times with each of the strains. The results represent average values.

The velocity of dehydrogenation of the donators by treated bacteria is expressed in arbitrary units related to the velocity of dehydrogenation of the same donators by normal, untreated bacteria, which was taken as 100.

RESULTS

(1) *The dehydrogenating enzymes of* E. typhosa. The bacteria were grown on agar slants of approximately equal size. The cells of each agar tube, after washing, were suspended in 1·5 ml. saline. The results are classified in the following manner:

Very strong donators: Complete dehydrogenation after 0–15 min.

Strong donators: Complete dehydrogenation after 16–60 min.

Weak donators: Complete dehydrogenation after > 60 min.

Not activated: No dehydrogenation.

All experimental series in which the control tube without donator showed dehydrogenation within 6 hr. were rejected; this occurred only rarely. The results obtained with the strain H 901 are given in Table 1.

It is noteworthy that hardly any difference was found between the strains H 901, O 901 and Vi 2, although these strains differed in their serological properties.

(2) *The effect of ultra-violet irradiation.* In the following experiments the influence of ultra-violet irradiation on some dehydrogenating enzymes of the strain H 901 was studied. A thin layer of washed bacteria was irradiated in open Petri dishes at 50 cm. distance from a quartz lamp; the dehydrogenating activities were compared, after irradiation of 1, 2, 4, 6 and 10 min., with the activities of non-irradiated portions of the same suspension. The results (Table 2) show a diminution of the dehydrogenating activity proportional to the time of exposure. The dehydrogenases for acids were more affected than those for alcohols and sugars.

To exclude the possibility that chemical changes occurring as the result of irradiation in the exposed suspensions had indirectly acted on the ferments, the following experiment was performed. A bacterial suspension was divided into four equal portions, of which one remained untreated, the second was irradiated, the third was irradiated and washed

Table 1. *Substrates activated as hydrogen donators by* E. typhosa (H 901)

Very strong donators	Strong donators	Weak donators	Not activated
Formic acid	Lactic acid	Acetic acid	Oxalic acid
Pyruvic acid	Succinic acid	Propionic acid	*l*-Tartaric acid
Mannitol	*d*-Tartaric acid	Malic acid	Maleic acid
Sorbitol	Ethanol	*dl*-Tartaric acid	Citric acid
Glucose	Glycerol	Fumaric acid	Methanol
Fructose	Arabinose	Xylose	Erythritol
Galactose	Melibiose	Sucrose	Adonitol
Mannose	Trehalose	Melezitose	Dulcitol
Maltose	*d*-Alanine	Raffinose	Inositol
	dl-Alanine	Arginine	Rhamnose
	Serine	Tryptophan	Sorbose
	Aspartic acid	Proline	α-Methyl glucoside
	Glutamic acid		Lactose
	Lysine		Glycine
	Asparagine		*d*-Leucine
	Histidine		*l*-Cystine
			Phenylalanine
			l-Tyrosine
			Urea
			Creatine
			Creatinine
			Guanidine

Table 2. *The effect of ultra-violet irradiation on certain dehydrogenases of* E. typhosa

		Irradiation time (min.)					
		0	1	2	4	6	10
		Relative dehydrogenating velocities (normal untreated bacteria = 100)					
Donator							
Acids (formic, pyruvic, glutamic, *d*-alanine)	Range	100	68–80	63–70	35–62	24–53	12–29
	Mean	100	75	66	52	43	21
Alcohols and sugars (mannitol, sorbitol, glucose, fructose)	Range	100	78–100	70–89	64–80	58–67	37–44
	Mean	100	88	84	71	60	41
Total average of donators tested		100	82	75	62	51	31

by centrifugation; to the fourth the supernatant fluid of the third portion was added. Equal reduction rates were noted in the first and fourth specimens on the one hand, and in the second and third on the other. From these results it may be concluded that ultra-violet irradiation acts directly on the dehydrogenating mechanisms of the cells.

(3) *The effect of salt solutions.* The influence of NaCl solutions of varying concentration was tested on eight dehydrogenases (formic and pyruvic acids, mannitol, sorbitol, glucose, fructose, *d*-alanine, glutamic acid) of the strain H 901. Equal amounts of washed bacteria were suspended in NaCl solutions of varying concentrations, and after 4 hr. their dehydrogenating properties were tested. From the results (Table 3) it follows that: (*a*) dehydrogenation in distilled water is slightly faster than that in 0·9 % NaCl; (*b*) hypertonic NaCl solutions suppress the dehydrogenating mechanisms, the inhibition increasing with increasing salt concentration; (*c*) the dehydrogenase for formic acid showed exceptional resistance and was influenced only by the highest NaCl concentration.

Table 3. *The effect of various* NaCl *concentrations on certain dehydrogenases of* E. typhosa

Donator		NaCl concentration (%) 0	0·9	3·0	5·0	7·0	10·0	15·0	30·0
		Relative dehydrogenating velocities							
Formic acid		100	100	100	100	100	100	100	67
Other donators (pyruvic acid, mannitol, sorbitol, glucose, fructose, glutamic acid, *d*-alanine)	Range	99–141	100	70–92	61–92	27–75	5–35	6–17	0·5–3
	Mean	112	100	83	78	53	21	9	2

(4) *The effect of freezing and thawing.* Repeated freezing and thawing considerably alter cell integrity. In our experiments a washed suspension of *E. typhosa* was frozen in an ice + salt mixture and thawed in a water-bath at 40°. This procedure was repeated ten times, the activity of the suspension being tested, for 14 donators (see Table 4), after every second freezing and compared with that of an untreated portion of the same suspension. In other experiments the number of surviving bacteria was determined. The examination carried out on the strains H 901 and O 901 yielded, essentially, the same results for both types of bacteria and, therefore, only the results obtained with H 901 are given here. A gradual decrease in the activity of almost all dehydrogenases was observed, which increased with each freezing. It is noteworthy that, out of 14 dehydrogenating mechanisms tested, only one, that for formic acid, remained completely uninfluenced by ten freezings. A comparison of the death-rate of the bacteria with loss in dehydrogenating capacity showed that the former always outweighed the latter. A possible explanation is that in estimating the number of 'living' bacteria we actually determine only those which are able to multiply, and this ability involves many fermentative processes which, as a whole, are more sensitive to freezing than the mechanisms of dehydrogenation.

Table 4. *The effect of freezing and thawing on the survival and on certain dehydrogenases of* E. typhosa

Donators		No. of freezings 0	2	4	6	8	10
		Death-rate (%) 0	52	80	84	89	93
		Relative dehydrogenating velocities					
Formic acid		100	100	100	100	100	100
Other donators (lactic, pyruvic, succinic acids, sorbitol, mannitol, fructose, galactose, mannose, glucose, maltose, *d*-alanine, glutamic acid, asparagine)	Range	100	60–89	55–84	43–79	38–63	31–60
	Mean	100	78	68	55	47	40

(5) *The effect of autolysis.* The formic dehydrogenase is apparently not affected by the destruction of the cell on repeated exposure to freezing, i.e. it is not bound to the intact cell structure. Cell-free extracts of bacteria were therefore tested for their dehydrogenating properties. The extracts were prepared by autolysis. A heavy suspension of *E. typhosa* (strain H 901) in phosphate-buffer solution at pH 7·2 was kept at 37° for 5 days; it was then strongly centrifuged and the supernatant liquid was used for dehydrogenating experiments. Out of 12 substances which were found to be activated as strong donators in the intact cells (formic, lactic and pyruvic acids, mannitol, sorbitol, mannose, glucose, fructose, galactose, maltose, *d*-alanine and glutamic acid), lactic acid was the only one to be dehydrogenated by the autolysate.

The different methods of cell lysis used have, therefore, different effects. Although most of the dehydrogenases are affected, formic dehydrogenase remains uninfluenced by freezing and thawing, and lactic dehydrogenase is not destroyed by autolysis at 37°.

DISCUSSION

Dehydrogenating enzymes of *E. typhosa* have already been studied by v. Vasarhelyi (1935). His strains activated essentially the same substances as did ours, but there are a number of differences between the two sets of results: acetic, propionic, malic and tartaric acids, glycerol, sucrose, alanine and lysine were not dehydrogenated in v. Vasarhelyi's experiments, while rhamnose was dehydrogenated. Dehydrogenation of formic, lactic and succinic acids by *E. typhosa* was also demonstrated by Stickland (1929). It is interesting to compare our results with those obtained by other authors with other bacteria of the coli-typhoid group. Quastel & Whetham (1925) noted strong activating properties in *Esch. coli* for formic and succinic acids, glucose, fructose, galactose and mannitol. Aaron (1933), working with *Salmonella schottmuelleri* and using the same technique as ours, found a rapid dehydrogenation of lactic acid, mannitol, sorbitol, xylose, glucose, fructose and maltose; and Braun & Woerdehoff (1933) noted powerful activation of formic, lactic and pyruvic acids, glucose, fructose and maltose by *Shigella paradysenteriae* (Flexner). It is, therefore, evident that practically the same substances are dehydrogenated by the various bacteria of this family.

It is noteworthy that no significant difference could be detected in the dehydrogenating abilities of the *E. typhosa* strains O 901, H 901 and Vi 2. Their different antigenic structures are not correlated with any distinct dehydrogenating mechanism. Braun & v. Vasarhelyi (1940) have shown that different strains of *Proteus vulgaris* also activate the same substrates.

Hypertonic NaCl solutions retard and hypotonic solutions accelerate the dehydrogenating velocity of most enzymes: the former reduce by shrinkage the active surface whilst hypotonic solutions, by swelling the cells, enlarge the cell surface and thus accelerate the dehydrogenating activities. Formic dehydrogenase, however, is little affected by these changes.

Young (1929) found that freezing and thawing strongly inhibited the lactic and glucose dehydrogenases of *E. coli* and left almost unaffected the formic and succinic dehydrogenases. In Yudkin's (1937*b*) experiments glucose dehydrogenase was eliminated, while that for lactic acid remained intact. Young's statement concerning formic dehydrogenase of *Esch. coli* agrees with our results obtained on *E. typhosa*. In the experiments of this author too, the ability of bacteria to multiply was affected more than their dehydrogenating properties. Adler *et al.* (1938) prepared by repeated freezing and thawing a cell-free extract of *Esch. coli* which contained the dehydrogenases for lactic and glutamic acids. The reduced activity, caused by freezing and thawing, of the dehydrogenases other than formic dehydrogenase may be due to an injury either to the cell structure or to the mechanism of hydrogen transport. The latter possibility is suggested by Yudkin's (1933, 1937*a*) observations: with glucose and lactic dehydrogenases of *Esch. coli* the loss of activity on dilution of the bacterial suspension was very great owing to dilution of a coenzyme. The activity of the enzyme could be restored by the addition of suspensions of heated bacteria. Lysis of a cell is invariably accompanied by a dilution of its content, but in experiments to test the effect of this it was found that neither the addition of heated bacteria nor of yeast cozymase was able to restore the activity, after ten freezings, of the enzymes tested (those for pyruvic acid, mannitol, sorbitol, glucose, fructose, *d*-alanine and glutamic acid). In these instances, therefore, the enzymes appeared to be linked in some way to the structure of the cell. In fact, when a bacterial suspension was frozen ten times and afterwards centrifuged, all the dehydrogenases remained in the sediment, the supernatant fluid being completely inactive. Slightly different results, however, were recorded in our experiments with cell-free extracts obtained by autolysis. It is interesting to note that among 12 substances tested, which were found to be strong donators when activated by living bacteria, only lactic acid was dehydrogenated by our extract. It follows, therefore, that lactic dehydrogenase is in some way independent of the cell structure.

A cell-free lactic acid-dehydrogenating enzyme of *Esch. coli* has already been prepared by Stephenson (1928) and by Adler *et al.* (1938): Stephenson's method of preparation was similar to ours. A peculiar property of formic dehydrogenase, resembling that found by us, was also noted by Stickland (1929); after destruction of the cell structure by tryptic digestion almost all dehydrogenases except that for formic acid were destroyed.

SUMMARY

1. Fifty-nine substances were tested for their ability to be activated as hydrogen donators by *Eberthella typhosa*. The most active donators were formic and pyruvic acids, mannitol, sorbitol, mannose, glucose, fructose, galactose and maltose. No significant difference could be detected between the dehydrogenating abilities of the serologically different strains H 901, O 901 and Vi 2.

2. Ultra-violet irradiation of the cells suppressed the dehydrogenating activity. Dehydrogenases for acids were more affected than those for alcohols and sugars.

3. Hypertonic NaCl solutions inhibited the dehydrogenating mechanisms for almost all donators, hypotonic ones slightly accelerated them. Only formic dehydrogenase was nearly insensitive to osmotic changes.

4. Repeated freezing and thawing inhibited the activity of almost all dehydrogenases tested, but formic dehydrogenase remained uninfluenced by as many as ten freezings. The diminution of the dehydrogenating velocity was not due to the dilution of a coenzyme.

5. Cell-free extracts, prepared by centrifugation of autolyzed bacteria, contained lactic acid dehydrogenase.

6. It is concluded that most dehydrogenating systems are in some way linked to the cell structure. Only formic and lactic dehydrogenases are in some degree independent of the intactness of cell structure.

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The Determination of Starch in Apple Tissue

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In the determination of starch present in apple tissues by the use of an enzyme, the tissue must first be obtained in a finely divided state so that the starch can be easily extracted and hydrolyzed. It is noticeable in the literature on starch determinations in apples that difficulty is experienced in obtaining complete extraction of starch, and that different values may be obtained according to the method of extraction and the enzyme which is used for hydrolysis.

In 1926 Gerhardt was able to extract starch quantitatively with boiling water from the ethanol-insoluble residue of dried apple tissue after removal of the substances soluble in 10% ethanol.

Widdowson (1932) records that she was unable to extract all the starch from apple tissue, unless the ethanol-insoluble residue was previously extracted, with a 1% K-oxalate solution, to remove pectin. Even after this treatment the residue had to be thoroughly ground in a mortar before complete hydrolysis of the starch by takadiastase could be effected.

Denny (1934) found it necessary to extract the ethanol-insoluble residue several times with ethyl ether and then to grind the dried tissue to a powder. After grinding the powder with water in a mortar he was able to determine starch quantitatively with takadiastase. He also describes a method based on the preliminary extraction of the starch in hot concentrated $CaCl_2$ solution, which, he states, gave true values for starch.

In 1936 Hanes determined starch in ethanol-extracted apple tissues by first rendering the fibre soluble by treatment with ethanolic HCl, and then hydrolyzing with β-amylase, which is specific for starch. His starch determinations were made on a set of ten apple residues supplied by Dr Kidd from an experiment with Bramley's apples, for which the authors had already obtained values by hydrolysis with takadiastase. Comparison of the results suggested the presence of a fraction hydrolyzable by takadiastase, but not by β-amylase.

The authors have not experienced difficulty in carrying out complete hydrolysis with takadiastase;

this may be due to the pre-treatment of the apples. The present investigation was carried out during 1936–7 in order to make further comparison of the starch values obtained by hydrolysis with takadiastase and β-amylase.

METHODS

The apples used were four pickings of Worcester Pearmain which were gathered on (i) 18 July, (ii) 29 July, (iii) 15 August and (iv) 2 September 1935, and stored at 10°, and which formed part of an experiment conducted by Drs Kidd and West in this laboratory. All samples of fruit for analysis were frozen to −20°, and then ground in the frozen state to a fine powder, which was weighed out in samples of 100 g. and kept at −20° in covered jars until required. The powder was extracted for 4 hr. with boiling 80% ethanol, and the insoluble residue dried to constant weight at 100°. The residue was then in a finely divided condition which crumbled to a fine powder when rubbed between the fingers.

Starch was determined in the ethanol-insoluble residue by the following methods.

Method 1. *Ethanol-insoluble residue treated with takadiastase.* This method is based on Widdowson's method (1932), in which the samples of the insoluble residue were digested with water in a boiling water-bath, and the starch hydrolyzed with takadiastase. Reducing sugar was determined by Maskell's (1935) modification of Shaffer & Hartman's (1921) micro-method, and related to the reducing power of a pure hydrolyzed apple starch. A blank was carried out on the takadiastase solution alone.

Method 2. *Ethanol-insoluble residue shaken with* 1% *(w/v) potassium oxalate solution, and then hydrolyzed with takadiastase.* The insoluble residue was shaken with 1% K-oxalate solution for 5 hr. in a mechanical shaker as described by Widdowson (1932). The residue was then filtered and washed free from oxalate with cold water, and then hydrolyzed with takadiastase as in method 1. The K-oxalate solutions were all tested with I_2 solution, and any colour reactions were recorded.

Method 3. *Ethanol-insoluble residue hydrolyzed with β-amylase.* The β-amylase was kindly supplied by Dr C. S. Hanes. The ethanol-insoluble residue was rendered soluble by treatment with ethanolic HCl, and then hydrolyzed with β-amylase, as described by Hanes (1936). The reducing power of the hydrolyzed starch was determined by the same method as in (1).

RESULTS

The values obtained by these methods are shown in Table 1, together with the colours produced by treating the K-oxalate extracts with I_2 solution in method 2.

DISCUSSION

Values for starch obtained by methods 2 and 3 show good agreement, but values by method 1 are much higher. This indicates that takadiastase hydrolyzes a substance or substances other than starch in the residue. The K-oxalate pre-treatment evidently removes this substance or substances, which may be called the 'non-starch' fraction.

When the K-oxalate filtrates were tested with I_2 solution the colours changed from deep blue, in the samples stored only a few days, to mauve or red-brown, in samples stored approximately 15 days; after 15 days no colour was obtained. A similar result was obtained by shaking the residue with cold water. Hydrolysis of the aqueous filtrate with takadiastase gave a reducing substance. Pure apple starch subjected to the same treatment with K-oxalate did not give any colour with I_2 solution. The 'non-starch' fraction, extractable with cold water or 1% K-oxalate solution, is therefore responsible for the colour reaction with I_2, and suggests the presence of dextrins.

Denny (1934) determined starch in plant tissues by takadiastase as described in method 1, and compared results with those obtained by extracting the starch with hot concentrated $CaCl_2$ solution, precipitating the starch with I_2, removing the I_2, and hydrolyzing the purified starch with takadiastase. He found that the first method gave values higher than the second, and assumed that the latter gave the true starch. He called the difference between the values obtained by the two methods 'the non-starch fraction hydrolyzable by takadiastase', but did not identify this fraction.

Widdowson (1932) examined the ethanol-insoluble residue from Bramley's Seedling apples for dextrin, by extracting with water at 38° for 24 hr., filtering, and treating the filtrate with I_2 solution, but did not obtain any colour. She also hydrolyzed the filtrate with takadiastase, but did not obtain any appreciable reducing activity, and therefore concluded that there is never more than a trace of dextrin in Bramley's apples. It is suggested that Widdowson's failure to obtain any coloration with I_2 may be due to the same cause that prevented complete extraction of the starch with water, namely, that the fibre was not in a sufficiently disintegrated condition to permit the penetration of the solvent. It appears that when the apple is frozen and ground, and kept frozen at −20°, the material of the cell wall may be broken down by freezing, and consequently the ethanol-insoluble residue is more permeable to the action of the solvent.

Gerhardt (1926) was able to extract from 0·1 to 0·59% of dextrin from apple tissue. He dried the apple tissue in a partial vacuum (50 mm. pressure) at 70° and ground the dried tissue in a mill until the powder would pass through a 100-mesh sieve. After removal of sugars, he extracted the residue with 10% ethanol at 50° for 30 min., evaporated the ethanol, clarified the solution, hydrolyzed with 2·5% HCl (approx. 0·7 N [?]), and calculated the reducing substances as dextrins.

Hence, there is some evidence that dextrins are present in apples at certain stages of growth.

Table 1. *Starch determinations on four pickings of Worcester Pearmain apples*

(Results as g./100 g. of fresh weight.)

Date of picking	Average size of fruit (g.)	Days in store (at 10°)	Hydrolysis by: Takadiastase (g./100 g.)	Hydrolysis by: Takadiastase after extraction with K-oxalate (g./100 g.)	Hydrolysis by: β-amylase (g./100 g.)	Colour obtained by addition of I_2 solution to the K-oxalate extract
18. vii. 35	25·6	0	2·09	1·92	—	Blue
		2	2·00	1·72	—	,,
		4	1·73	1·66	—	Mauve
		4	1·54	1·42	—	,,
		8	1·38	1·08	—	Red-brown
		12	0·97	0·79	—	None
		20	0·23	0·10	—	,,
		20	0·26	0·10	—	,,
		30	0·16	0·05	—	,,
		40	0·16	0·05	—	,,
		50	0·12	0·04	—	,,
		60	0·17	0·04	—	,,
		(at 10°)				
29. vii. 35	37·2	0	2·26	2·09	—	Blue
		2	2·20	1·98	—	,,
		4	2·00	1·74	—	,,
		4	1·70	1·59	—	,,
		8	1·29	1·21	—	None
		12	0·79	0·65	—	,,
		19	0·22	0·08	—	,,
		19	0·19	0·07	—	,,
		30	0·15	0·03	—	,,
		40	0·16	0·02	—	,,
		50	0·15	0·02	—	,,
		60	0·17	0·02	—	,,
		(at 10°)				
15. viii. 35	61·8	0	2·48	2·17	2·14	Blue
		2	2·58	2·11	2·23	,,
		5	2·05	1·66	1·69	,,
		8	1·49	1·07	1·19	,,
		11	0·90	0·65	0·62	,,
		14	0·64	0·43	0·38	Mauve
		18	0·31	0·15	0·13	None
		21	0·26	0·08	0·07	,,
		25	0·19	0·03	0·03	,,
		28	0·17	0·03	0·03	,,
		32	0·17	0·02	0·03	,,
		65	0·17	0·02	0·02	,,
		127	0·17	0·02	0·02	,,
		(at 1°)				
15. viii. 35	61·8	0	2·48	2·17	2·14	Blue
		5	2·10	1·81	1·72	,,
		11	1·55	1·19	1·19	,,
		16	1·22	0·88	0·94	,,
		22	0·83	0·58	0·54	Mauve
		28	0·29	0·11	0·11	None
		34	0·48	0·25	0·25	,,
		40	0·33	0·17	0·15	,,
		46	0·22	0·08	0·06	,,
		51	0·23	0·04	0·05	,,
		56	0·19	0·03	0·03	,,
		63	0·20	0·03	0·02	,,
		69	0·18	0·02	0·03	,,
		204	0·16	0·02	0·03	,,
		(at 10°)				
2. ix. 35	72·4	0	1·79	1·52	—	Blue
		2	1·71	1·53	—	,,
		4	1·39	1·22	—	,,
		4	1·52	1·19	—	,,
		8	0·72	0·60	—	Mauve
		12	0·41	0·31	—	None
		19	0·16	0·06	—	,,
		19	0·14	0·06	—	,,
		30	0·09	0·03	—	,,
		40	0·10	0·04	—	,,
		51	0·06	0·02	—	,,
		60	0·09	0·02	—	,,

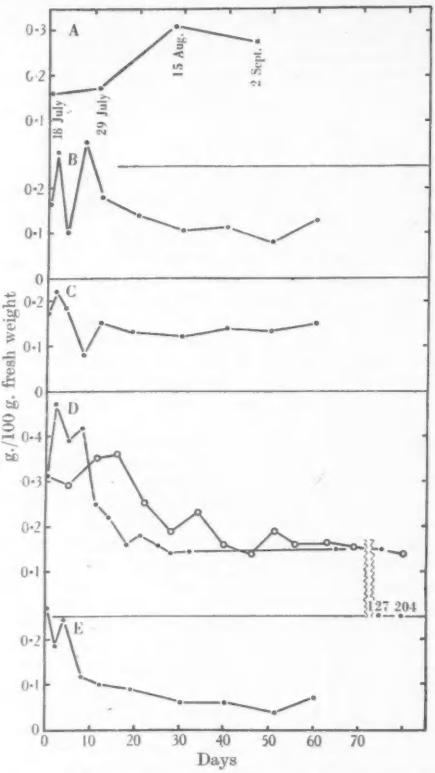

Fig. 1. Changes in the non-starch fraction of Worcester Pearmain apples. A. With growth from 18. vii. 35 to 2. ix. 35. B. First pick gathered on 18. vii. 35 and stored at 10°. C. Second pick gathered on 29. vii. 35 and stored at 10°. D. Third pick gathered on 15. viii. 35 and stored at 10° •—•, and at 1° o—o. E. Fourth pick gathered on 2. ix. 35 and stored at 10°.

The difference in the values obtained by methods 1 and 2 (or 3) is a measure of the 'non-starch' fraction, and Fig. 1 shows the changes in this fraction during growth and storage.

This fraction, at the time of picking, increases in value up to 15 August, and then shows a slight fall on 2 September, which coincides with the increase and decrease of true starch. During the first few days of storage, there is a slight increase in 'non-starch' in the first and second picks, a definite increase in the third pick, but an immediate fall in the fourth pick. In each gathering, the non-starch fraction falls to an approximately constant value after the disappearance of true starch.

As there is no increase in true starch during storage, it is possible that the 'non-starch' is a degradation product. The greatest increase in 'non-starch' is found where true starch is at its maximum, that is, on 15 August.

SUMMARY

1. The freezing of apple tissue produces a fibre in which all the starch can be hydrolyzed with takadiastase.

2. If the ethanol-insoluble residue from frozen apple is shaken with 1 % potassium oxalate solution, a 'non-starch' fraction hydrolyzable by takadiastase is extracted. The evidence suggests the possibility of the presence of dextrins.

3. The starch determinations by Hanes's method using β-amylase give similar values to those obtained by the action of takadiastase on the residue after treatment with potassium oxalate. These values are assumed to be true starch.

4. The 'non-starch' fraction falls during storage to an approximately constant figure. The fall takes place during the period of true starch hydrolysis, and the substances giving colours with iodine are also present in the fraction during this period.

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Nomograms for Manometer Constants

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By the use of the nomograms described below, the constants of Warburg (constant-volume) and Barcroft (differential) manometers can be obtained in a small fraction of the time required for calculation by the usual methods.

I. THE WARBURG MANOMETER

The constant of the Warburg manometer is given by the expression

$$k = \frac{\frac{273}{T} v_G + v_F \alpha}{P_0}, \qquad (1)$$

where T is the absolute temperature, v_G and v_F are the volumes (in μl.) of the gas and liquid phases in the vessel, α is the solubility (Bunsen coefficient) of the particular gas being measured, and P_0 is the number of mm. of manometric fluid equivalent to 760 mm. Hg.

In practice it is more convenient to express v_G and v_F in ml. instead of μl.; this may be done without changing the value of k if P_0 is expressed in m. instead of mm. When Brodie solution is used the value of P_0 may then be taken as 10.

A nomogram to take account of all the variables in the above expression would be somewhat complex, but in practice P_0 is constant, and if T is also taken as constant, α for a particular gas is also constant and the expression is then represented by a very simple form of nomogram. This will apply to only one particular temperature, but most experimental work is carried out at a constant temperature, and as scales for two or more temperatures may be drawn on the same nomogram, most requirements will be met.

v_G is obtained by subtracting v_F from the total volume V of the vessel, which is usually marked on the instrument, and it is convenient to make the nomogram perform the subtraction automatically. Substituting $v_G = V - v_F$ in equation (1), we get

$$k = \frac{273}{TP_0} V - \left(\frac{273}{TP_0} - \frac{\alpha}{P_0}\right) v_F. \qquad (2)^*$$

For O_2 at 37°, $\alpha = 0{\cdot}024$ and we have therefore

$$k = 0{\cdot}0881 V - 0{\cdot}0857 v_F. \qquad (3)$$

For CO_2 at 37°,

$$k = 0{\cdot}0881 V - 0{\cdot}033 v_F. \qquad (4)$$

The theory of nomograms corresponding to expressions of this type is given by Allcock & Jones (1941, p. 42). The nomogram consists of three parallel straight lines carrying scales of V, v_F and k. It is used by placing a straight-edge so that it passes through the correct values of V and v_F; the edge then cuts the k scale at the required value of k. Fig. 1 shows the nomogram for Warburg manometer constants, with scales for both O_2 and CO_2 constants at two different temperatures. By its use the values of the constants may be obtained in a few seconds.

A strip of celluloid or other transparent material, with a straight line scribed on its under surface, will be found preferable to the edge of a ruler, since it does not obscure any part of the scales.

The nomogram may be constructed by making use of the mathematical theory given by Allcock & Jones, but it is simpler and, if care is taken, equally accurate, to use the following graphical method. A straight line is drawn near the left-hand side of the paper and marked off with a uniform scale of V values, in any convenient scale units so that the scale covers the range of values likely to be encountered. At any convenient distance another straight line is drawn parallel to the first and marked off with a scale of v_F in any convenient units, but running downwards. Two values of k, fairly well separated, are then chosen arbitrarily; for each value of k two convenient values of v_F are selected, and the corresponding values of V are calculated (e.g. from equation (3)), as in Table 1. Four pencil

Table 1. *Corresponding values of k, v_F and V*

k	v_F	V
2·0	1	23·68
2·0	3	25·63
1·5	2	18·97
1·5	4	20·92

any value of v_F can then be read off from the appropriate graph. It is necessary to draw a separate graph for each manometer, for O_2 and CO_2, and for each temperature. The labour, however, is lightened by the fact that the graphs are straight lines and (for a given gas and temperature) parallel to one another, so that parallel rulers may be used. The nomogram seems to be a more convenient method.

* After this paper was sent to press, my attention was drawn to a note by MacLeod & Summerson (1940) in which equation (2) is given and is made the basis of a graphical method (not a nomogram) for determining Warburg manometer constants. In this method a separate graph is drawn for each manometer vessel, by plotting the values of k against the corresponding values of v_F. The value of k for

lines are then drawn to join the corresponding pairs of V and v_F values and a straight line for the k scale is drawn through the two points of intersection. the scale to the ends of the line. This is done most easily by the usual method of drawing a pencil line at any convenient angle to pass through one of the

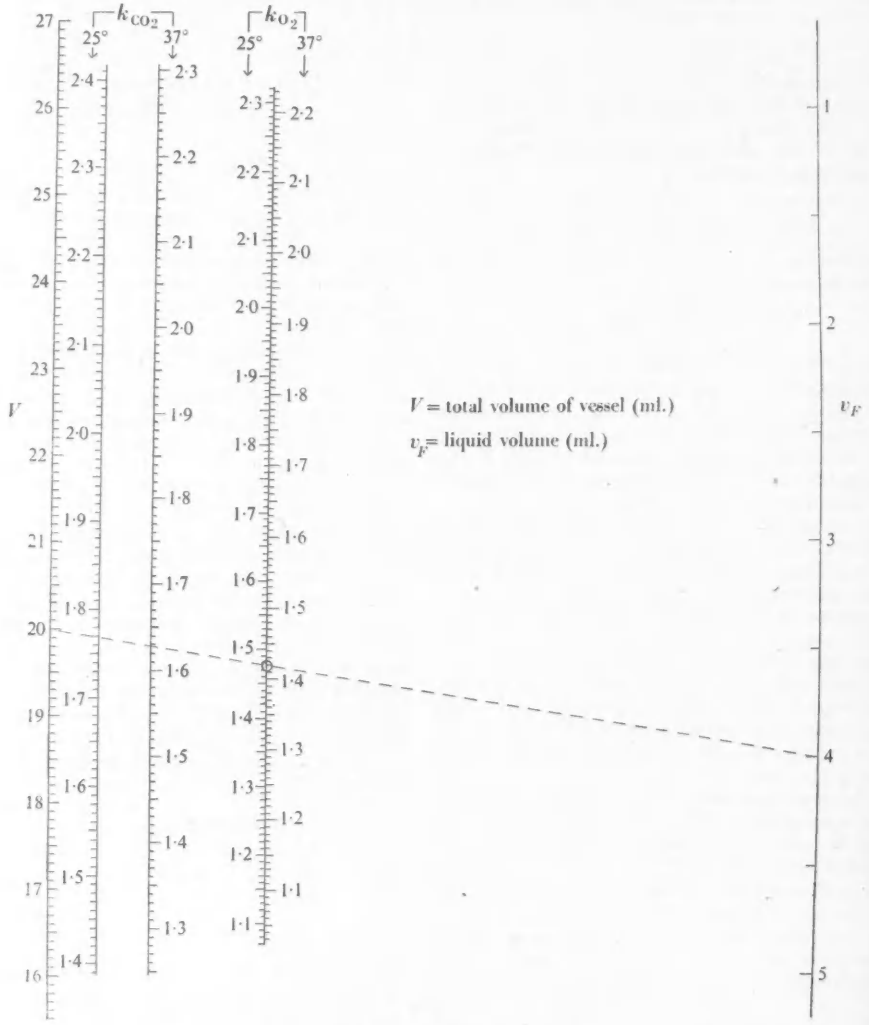

Fig. 1. Warburg manometer constants.

This should be found to be accurately parallel to the other two scales. The scale of k values is then set off along this line by dividing the distance between the two points of intersection into the correct number of equal parts, in this case 5, and continuing points of intersection, marking it off in inches or other convenient units, drawing a line from the fifth mark to the other point of intersection, and drawing parallel lines from the other marks to cut the k scale. This will divide the k scale into intervals of 0·1.

Each interval may conveniently be divided into 10 parts to give the 0·01 graduations as follows. A pencil of 11 equally spaced lines diverging from a point is drawn on a separate piece of paper, and is cut off with scissors at right angles to the centre line at a point where the total width of the pencil is equal to the distance between the 0·1 graduations of the k scale. If the cut edge is then placed in contact with the k scale, the 0·01 graduations may easily be inserted accurately. The same pencil of lines may be used for several different scales, if they are taken in the proper order, by cutting off the appropriate portions successively. As pointed out by Allcock & Jones (1941), this device may frequently be used for non-uniform scales by making the cut at an appropriate angle.

If desired, a check on the accuracy of the construction of the nomogram may be made, using the mathematical theory as follows. If the distance between the V and v_F scales is 18 cm., if 1 unit of V occupies 2 cm. on the scale and 1 unit of v_F occupies 5 cm., and if the nomogram represents equation (3), the k scale will be situated at a distance of $18\left(1+\frac{5}{2}\frac{0{\cdot}0881}{0{\cdot}0857}\right)$ cm. or 5·04 cm. from the V scale, and 1 unit on the k scale (i.e. 10 of the 0·1 intervals) will occupy $\frac{2\times5}{2\times0{\cdot}0857+5\times0{\cdot}0881}$ or 16·35 cm.

In some laboratories the vertical type of manometer has been abandoned in favour of a type of Warburg manometer in which the U-tube and scale are tilted backwards at an angle θ with the vertical, as in the Barcroft manometer, for convenience in reading and handling. The constant is then given by the expression

$$k=\cos\theta\,\frac{\frac{273}{T}v_G+v_F\alpha}{P_0}. \qquad (5)$$

The same nomogram applies, except that the values shown on the k scale must all be cos θ times those shown in Fig. 1.

II. THE BARCROFT MANOMETER

The case of the Barcroft (differential) manometer is more complicated, but the saving of time by using the nomogram method is even more marked. By this method it is easily possible to calculate a constant within 30 sec.

The constant for this manometer is given by the expression (Dixon, 1943, p. 27)

$$k=\left(\cos\theta+\frac{AP_0}{2v'_G}\right)\left(\frac{\frac{273}{T}v_G+v_F\alpha}{P_0}+\frac{A}{2}\frac{273}{T}\right), \qquad (6)$$

where A is the cross-section of the U-tube in sq.mm., v'_G is the volume of the gas phase in the left-hand (control) vessel, and the other symbols have the same meanings as in the previous section.

A single nomogram to represent the whole of this expression would be inconveniently complicated, and it is preferable to use two separate nomograms to obtain the two expressions in brackets, afterwards multiplying them together. Calling the expression in the first brackets C, and that in the second brackets D, we have

$$k=CD. \qquad (7)$$

C is identical with the 'correction factor C' (Dixon, 1937), used in the measurement of CO_2 retention, and the nomogram is also useful for its determination.

It appears that equation (6) involves seven independent variables, but as before several of these may be taken as constant. θ remains fixed for a given installation, P_0 and T may again be considered as constants and α is then fixed for a given gas. We are left with the two variables A and v'_G determining C, and with the three variables A, v_F and v_G (or V) determining D.

If θ is 15° and P_0 (for kerosene) is 13·1 m., we have

$$C=0{\cdot}966+6{\cdot}55\frac{A}{v'_G}. \qquad (8)$$

The corresponding nomogram consists of two parallel scales for A and v'_G and a non-uniform C scale which is not parallel to the others, but passes through their respective zeros. It is shown in Fig. 2*a*. It is readily constructed by the graphical method of the previous section, using two pairs of intersecting lines, drawn between corresponding values calculated from equation (8), to fix the position of the C scale. Any convenient positions and units may be chosen for the A and v'_G scales, provided that they are parallel, cover the required range of values, and run in opposite directions. As the C scale is non-uniform, it is most easily graduated by drawing lines corresponding to a number of selected values of C between A and v'_G values obtained from equation (8). The scale is a very open one, with few graduations, and gives a much greater degree of accuracy than is needed.

In the nomogram for D it is convenient to make the calculation of v_G automatic by substituting $v_G=V-v_F$, as in the previous section. This gives

$$D=\frac{273}{TP_0}V+\frac{273}{2T}A-\left(\frac{273}{TP_0}-\frac{\alpha}{P_0}\right)v_F. \qquad (9)$$

For $T=37°$ and for O_2, taking the same value for P_0,

$$D=0{\cdot}0672V+0{\cdot}440A-0{\cdot}0654v_F, \qquad (10)$$

and for CO_2

$$D=0{\cdot}0672V+0{\cdot}440A-0{\cdot}0252v_F. \qquad (11)$$

As these expressions for D contain three independent variables, instead of two as in the previous cases, a single alinement nomogram cannot be used. Two alternative types of nomogram are available, namely a 'set-square' nomogram, as shown in Fig. 2*b*, or a double-alinement nomogram, as shown

in Fig. 2*c*. The writer prefers the set-square type, but both have their advantages, and as some have expressed a preference for the second type, both are given here.

To use the nomogram in Fig. 2*b*, a 90° set-square (the corner of a sheet of paper is usually sufficiently accurate) is placed so that one edge passes through V and A and the other edge passes through v_F. The

against it, so as to form a T-square. This is the case, for example, for all values of v_F when $V = 34$ and $A = 0{\cdot}8$.

The best way of using this nomogram is to have a sheet of celluloid or thin perspex with its under surface scribed with two straight lines meeting accurately at 90° so as to form a T. With this, no difficulties will arise, and no part of the scales will

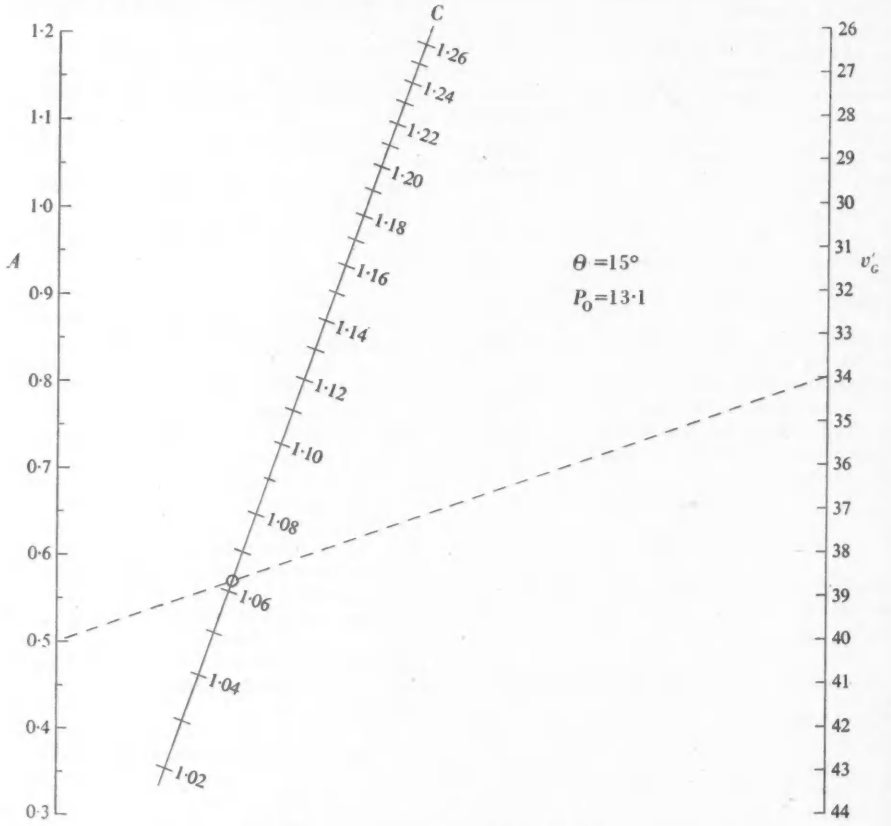

Fig. 2*a*. Barcroft manometer constants. I.

value of D is then indicated by the point at which the second edge passes through the D scale. The square may be placed on the nomogram in any position, provided only that one side passes through V and A and the other through v_F and D. With a few combinations of values (such that the corner of the square lies between the V and A scales or between the v_F and D scales) it will be found that this can only be done if one or other of the sides of the square is prolonged by placing a straight-edge

be obscured as with a set-square or sheet of paper. In reading the intermediate divisions of the D scale, it is necessary to bear in mind that this scale runs from right to left.

The construction is as follows. A straight line is drawn in any convenient position near the left-hand side of the paper and is marked with a uniform scale of V values, using any convenient units. A line for the A scale is drawn parallel to the V scale at a convenient distance from it. The positioning of the

A scale along this line is arbitrary, but should be chosen so that the centre of the useful range of A values comes fairly near the centre of the V scale. The actual units of the A scale, however, cannot be arbitrarily chosen, but are fixed by the units chosen for the V scale. If the nomogram is to represent either equation (10) or equation (11), and 1 unit on the V scale occupies 2 cm., 1 unit on the A scale (or 10 divisions of 0·1) must occupy $2 \times \frac{0{\cdot}440}{0{\cdot}0672}$ or 13·1 cm. The A scale must run in a downward direction.

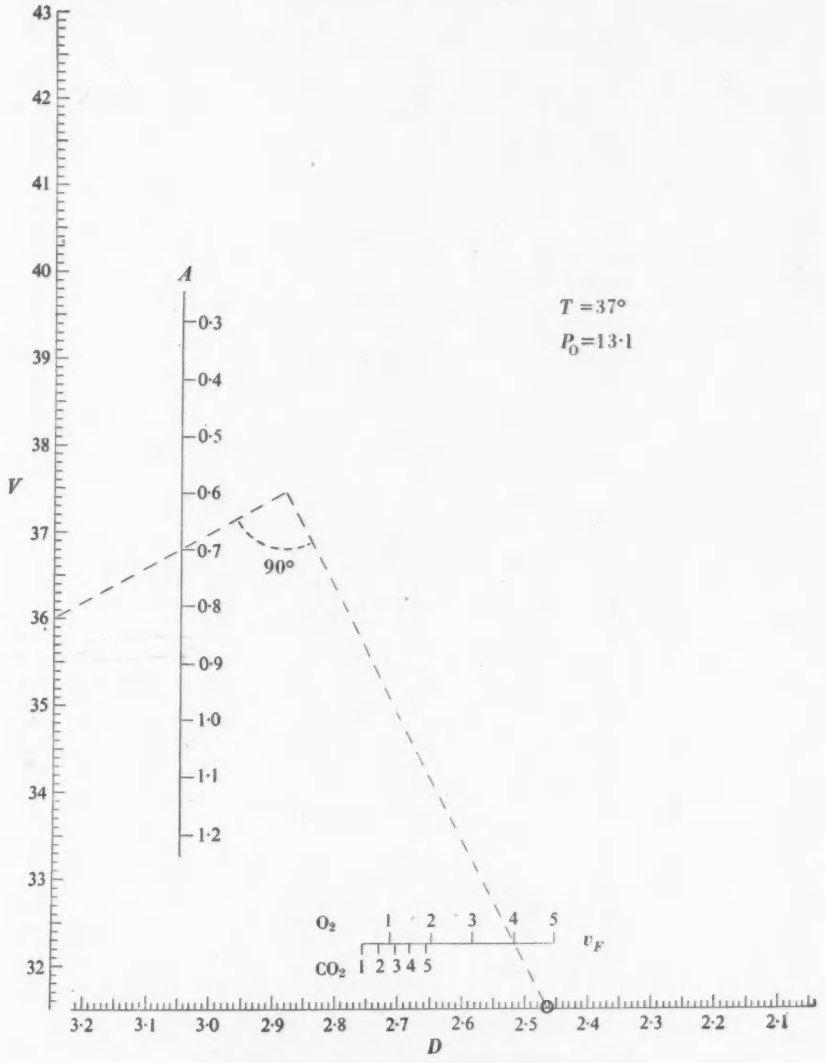

Fig. 2*b*. Barcroft manometer constants. II.

A line for the D scale is drawn in any convenient position accurately at right angles to the V and A scales, and a line for the v_F scale is drawn at a convenient distance away from the D line and parallel

to it. The placing of the v_F scale along this line is arbitrary, but controls that of the D scale, and it should be chosen so that the range of D values most likely to be required lies within the boundaries of the nomogram. The v_F scale units depend on the distances between the two pairs of scales, the constants of the equation, and the units chosen for the V scale. If the distance between the V and A scales is 3 cm. and that between the v_F and D scales is 1·5 cm., and if 1 V unit occupies 2 cm. and the nomogram is for equation (10), 1 v_F unit must occupy $2 \times \frac{0{\cdot}0654}{0{\cdot}0672} \times \frac{1{\cdot}5}{3{\cdot}0}$ or 0·973 cm. For CO_2 (equation (11)), 1 v_F unit must occupy $2 \times \frac{0{\cdot}0252}{0{\cdot}0672} \times \frac{1{\cdot}5}{3{\cdot}0}$ or 0·375 cm. The zeros of the v_F scales for O_2 and CO_2 must be placed to coincide if the same D scale is to apply to both. One unit on the D scale (or 10 divisions of 0·1) will occupy $2 \times \frac{1}{0{\cdot}0672} \times \frac{1{\cdot}5}{3{\cdot}0}$ or 14·88 cm.,

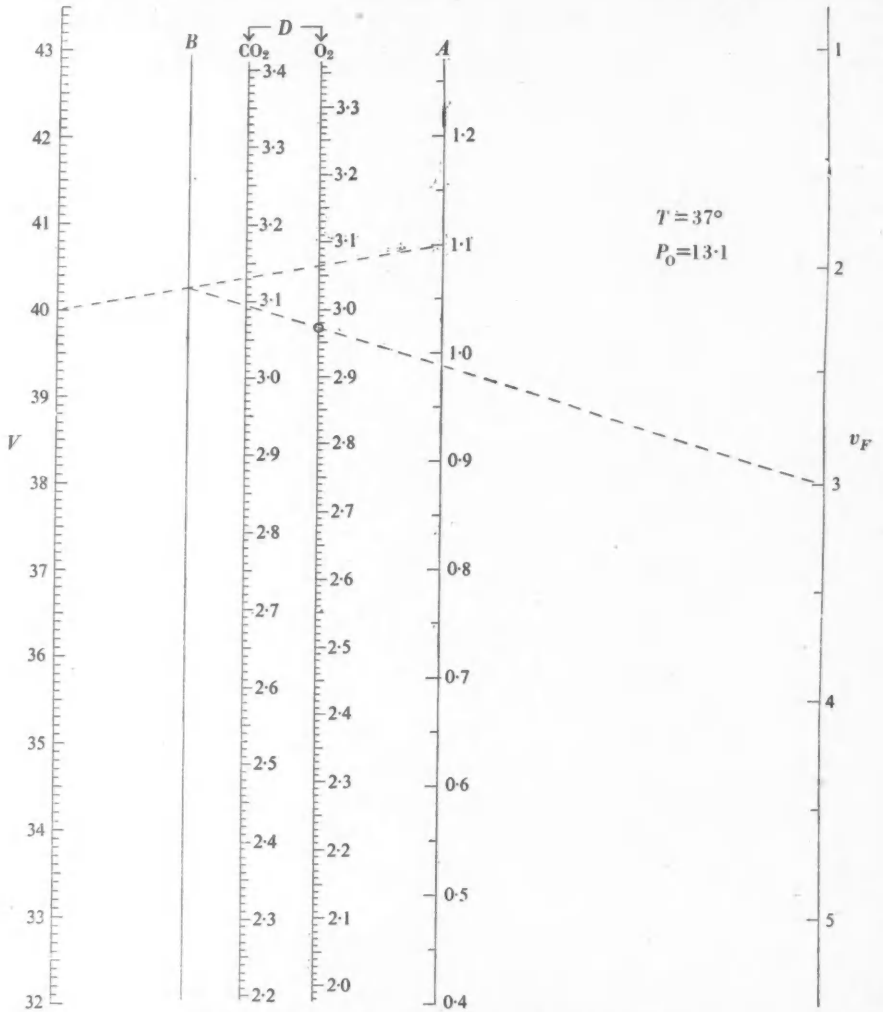

Fig. 2c. Barcroft manometer constants. II (alternative form).

and one setting of the square with known values of the variables, calculated from equation (10) or (11), is sufficient to fix its position. All the scales are uniform, and they may be divided by the methods given in section I.

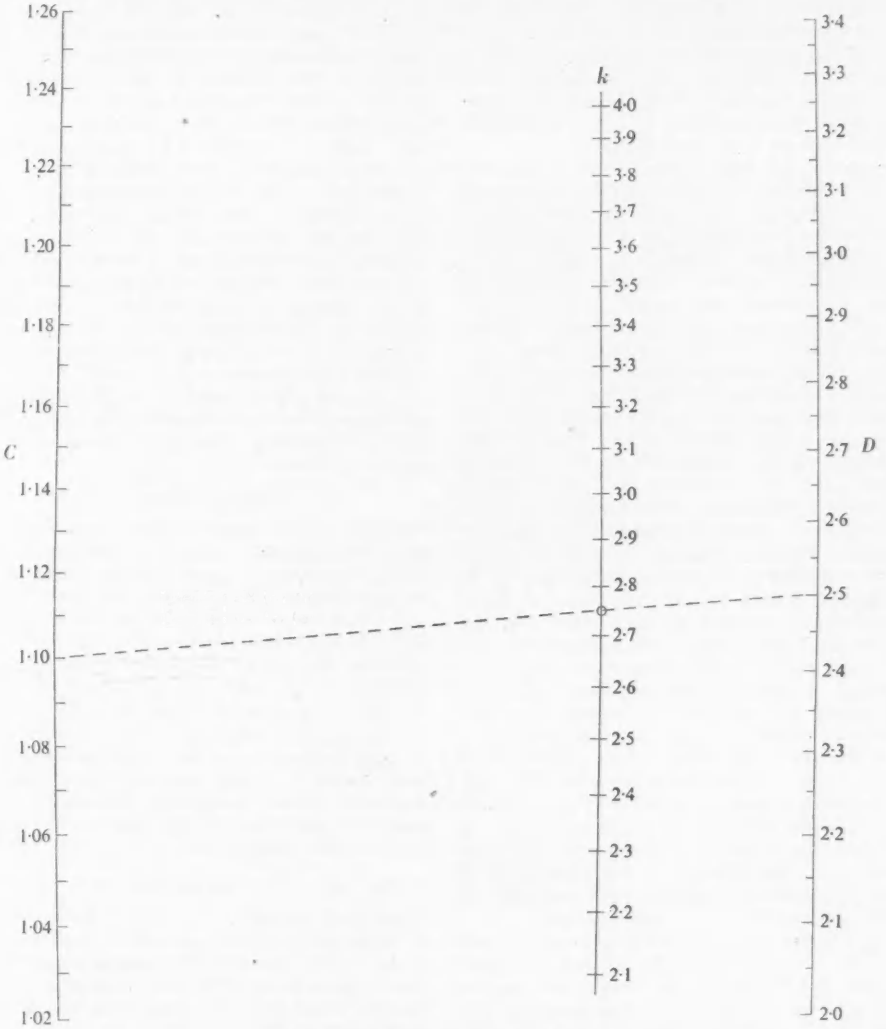

Fig. 2*d*. Barcroft manometer constants. III.

The double alinement nomogram for D (Fig. 2*c*) is used as follows. A straight-edge is placed so as to pass through A and V, a mark is made at the point where it cuts the reference line B, the straight-edge is then placed so as to pass through this mark and v_F, and the value of D read off at the point at which it intersects the appropriate D scale.

This nomogram is really a combination of two nomograms, each of the type shown in Fig. 1. B is actually the sum of the first two terms in equation (10) and it is determined from the V and A scales. The second setting gives the sum of B and the last term of the equation for D. It is unnecessary to

provide the B line with scale divisions, as it is used merely as an intermediate reference line.

The construction is carried out in two stages exactly according to the principles given in section I. The positions and units of the V, A and v_p scales may be chosen arbitrarily. The position and units of the V and A scales fix the position of the B line, which may be determined either graphically or by calculation as described earlier. The B and v_p scales then fix the D scales according to the same principles. All the scales are uniform and parallel.

In practice, this type is not so rapid in use as the set-square type and it requires more care and accuracy in setting. It is also slightly more confusing and there is some slight danger of taking the scales in the wrong order. Another disadvantage is that it is necessary to mark the nomogram and wear will be caused by erasure of the marks. If the marks are not erased each time, there is some danger of using a previous mark by mistake. On the other hand, its operation is somewhat easier to understand, and it has the advantage that the values for both the O_2 and CO_2 constants of a manometer can be read off at one setting. The accuracy of both types is all that can be desired, being of the order of 0·1 %.

To obtain the constant, the values of C and D obtained from their respective nomograms are multiplied together. This may be done with a slide rule, or if desired by a separate nomogram, as shown in Fig. 2*d*. This is an ordinary single-alinement nomogram, except that all three scales are logarithmic. This is most easily constructed by drawing two uniform and parallel scales of log C and log D, covering the required ranges of C and D, but with the figures only temporarily inserted in pencil. After completing the nomogram with the scale of log k, the actual values of C, D and k are inserted at the correct points to coincide with their logarithms, using a table of logarithms, and the pencil figures erased. Alternatively, the scales may be graduated by projection from a standard logarithmic scale, e.g. a slide rule. This nomogram should be made of reasonably large size, if the accuracy is to be comparable with that already obtained.

The nomogram for C is independent of temperature, but D changes with the temperature. As in the case of the Warburg manometer, scales for more than one temperature may be drawn on the nomogram for D. The double-alinement type (Fig. 2*c*) is less suitable for this than the set-square type, owing to the confusing effect of the larger number of parallel scales. In the set-square type, since the effect of temperature on the coefficients of the V and A terms is the same, as shown by equation (9), the same V and A scales can be used for both temperatures. The same D scale may also be used if a separate v_p scale is drawn for each temperature at suitably adjusted distances from the D scale. But as the v_p lines for the different temperatures lie very close together it is difficult to avoid overlapping, and it is preferable to draw a separate D scale and associated v_p scale for each temperature. As the placing of these scales is quite arbitrary, provided only that they are at right angles to the V and A scales and that the D and v_p scales retain their relative position, the pair of scales for the second temperature may be placed at any convenient position on the diagram, e.g. near the top, so that there is no risk of confusion between the scales for different temperatures.

It may be pointed out that for O_2 D varies inversely as T; this is not accurately the case for CO_2, owing to the large effect of temperature on its solubility.

III. OTHER MANOMETERS

Practically all the types of manometers now in use are covered by one or other of the nomograms given here. Equation (1) gives the constants of the constant-volume differential manometers of Dickens & Greville and of Summerson, as well as those of the Heatley micro-respirometer and Needham's Cartesian diver micro-respirometer. The nomogram of Fig. 1 can therefore be used for all these cases. Equation (6) applies also to the Dixon-Keilin manometer, and the nomograms of Fig. 2 are applicable. It must be noted, however, that the use of other manometric fluids generally requires a change in the scales. This is especially the case if one of the heavy liquids is used, as in the original Dixon-Keilin method.

SUMMARY

Nomograms are given by which the constants of Warburg (constant-volume) and Barcroft (differential) manometers may be determined accurately in a small fraction of the time required for calculation by other methods. Directions are given for their construction.

The nomograms in this paper are being published in booklet form by the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1., price sixpence.

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The Conditions Controlling the Production of Hyaluronidase by Micro-organisms Grown in Simplified Media

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The purpose of the present work was to design protein- and peptone-free media (to be called 'simplified media') in which various micro-organisms would grow and synthesize hyaluronidase. The antigenically specific enzymes thus produced could then be purified from the culture filtrates and examined. In this work it has been found that various groups of organisms have different nutritional requirements for the production of hyaluronidase. A study of the response of organisms to the inclusion of hyaluronate and its breakdown products in the medium has revealed that hyaluronidase preparations from various sources differ in the degree to which they hydrolyze hyaluronate. Brief preliminary reports of this work have already appeared (Rogers, 1944*b*, *c*).

METHODS

The amounts of hyaluronidase in the supernatant fluids from 16–18 hr. centrifuged cultures were determined by the mucin clot-prevention (M.C.P.) method (McClean, 1943) using twofold dilutions, and by the viscosity reduction method (McClean & Hale, 1941) as modified by McClean (1943) (this modification allows the accurate determination of streptococcal enzyme; Hale, 1944). A single batch of potassium hyaluronate was used as substrate for all the enzyme estimations. The enzyme titres of the samples, as assayed by either test, are recorded in the tables as comparisons with a dried preparation of testicular hyaluronidase of known potency. The mucin clot-prevention results are recorded (under M.C.P.) as reciprocals of the enzyme titres; viscosity reduction results are recorded as viscosity reduction units (under V.R.U.) (cf. McClean & Hale, 1941; McClean, 1943).

Bacterial-N was estimated by the micro-Kjeldahl method upon twice-washed deposits, centrifuged from 10 ml. of culture. Both the quantity of the enzyme produced and the amount of bacterial-N were determined in duplicate cultures for each modification of the medium. The results given in the protocols represent the mean of these two determinations. The amount of enzyme synthesized by unit mass of bacterial cells was calculated from the ratio: Mean V.R.U./Mean value for mg. bacterial-N per 10 ml. of culture. When enzyme synthesis by an organism was to be compared in a series of different media all the tubes were inoculated from the same seed culture.

Glucosamine estimations were made by the method of Elson & Morgan (1933) upon the samples of K hyaluronate hydrolyzed by treatment for 4 hr. with 4 N-HCl in sealed ampoules immersed in a boiling water-bath. Reducing sugars were estimated upon the enzymatically hydrolyzed K hyaluronate by Somogyi's (1933) modification of the Hartman-Shaffer alkaline copper reagent. Equimolecular mixtures of *N*-acetylglucosamine and glucurone were used as standards in the latter estimation, since neither of these substances alone brings about the degree of reduction of the reagent to be expected from an equivalent quantity of glucose.

Preparation of purified hyaluronate

Considerable degradation of the polysaccharide occurs when methods such as that described by Madinaveitia & Stacey (1944) are used; for this reason the method of purification employed in this work is described. The work of Meyer & Palmer (1936), Morgan & King (1943), and more recently of Blix & Snellman (1944, 1945) has revealed the necessity for using gentle methods of treatment if the physical properties of polysaccharides are to be preserved. This is emphasized by the results recorded in Fig. 1, which shows the deleterious influence of

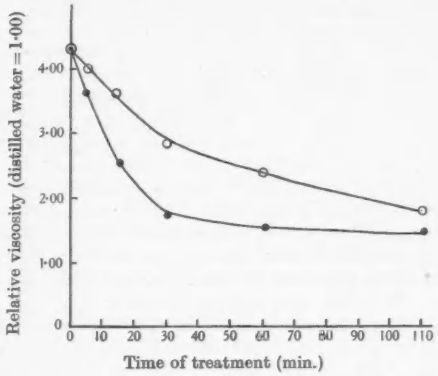

Fig. 1. The influence of heating upon the relative viscosity of solutions of crude potassium hyaluronate (0·5 g./100 ml.) adjusted to pH 7·0 o—o and 8·0 •—• and maintained at the temperature of a boiling water-bath.

maintaining, at the temperature of a boiling water-bath, sealed ampoules of crude potassium hyaluronate solution adjusted to pH 7·0 or 8·0. The method to be described yields a highly viscous product.

(1) 1½ vol. of ethanol saturated with potassium acetate and cooled to −10° were added to an aqueous solution at 0° of crude potassium hyaluronate (prepared by Glaxo Laboratories Ltd.) containing 0·5 g./100 ml. and adjusted to pH 9·0 (McClean, 1943). The fibrous clot which formed was collected on a stirring rod, transferred to a flask, washed thrice with ethanol, twice with ether and dried *in vacuo* at 90°. The dry product was dissolved and reprecipitated, once.

(2) The twice-precipitated material was ground in a mortar with 2 vol. of 90% (w/v) phenol solution (Morgan & Partridge, 1941), each equal to one-tenth of the volume of the original polysaccharide solution. The insoluble hyaluronate was then washed free of phenol with ethanol followed by ether and dried as before.

(3) The material from (2) was dissolved in sufficient water to give a 0·5 g./100 ml. solution. 10% by volume of a 50 : 50 mixture of *n*-butanol and chloroform was added to the solution, which was then shaken for 6 hr. The emulsion formed was separated by centrifuging for 2 hr. at 2000–3000 r.p.m.

(4) The supernatant from (3) was diluted with an equal volume of water, adjusted to contain 7 g./100 ml. NaCl to decrease its viscosity reversibly (Madinaveitia & Quibell, 1940) and shaken for 5 min. with kaolin; the latter was then removed by gravity filtration through fluted no. 2 Whatman papers over a period of 48 hr. The concentration of chloroform was maintained throughout to prevent bacterial growth.

(5) A few drops of toluene were added to the filtrate from (4) which was then dialyzed for 48 hr. against running tap water, when the viscosity of the solution became restored. The dialysate was adjusted to pH 9·0 and the hyaluronate precipitated by the addition of 2 vol. of ethanol and a trace of potassium acetate. The precipitate, removed by centrifuging and dried with absolute ethanol and ether, was equivalent to 20–30% of the initial crude product.

The final product gave solutions which varied from crystal clarity to slight opalescence. Biuret tests and the usual protein precipitants all gave negative results with solutions of the purified material. These tests are, however, comparatively crude for the examination of polysaccharides and no claim is made that the described method of preparation yields hyaluronate completely free from protein or polypeptide contaminants. Table 1 gives analytical figures for two batches of the purified material and for the crude samples from which they were prepared. The relative viscosity of a 0·1 g./100 ml. solution of batch E in distilled water was 3·70 at 34° as compared with 2·6 for a solution in buffer (2·3 g./100 ml.) of the better of the two preparations of Madinaveitia & Stacey (1944). The nitrogen content of the preparations was about 3·0 % of their dry matter, compared with 5·0% for those of Madinaveitia & Stacey (1944); Meyer & Palmer's (1936) ash-free preparations of hyaluronic acid contained about 3·3% N. Thus it may be said that the preparations used here were more likely to be free from protein, or from amino-acid-containing contaminants, than those of Madinaveitia & Stacey (1944), yet they were not grossly degraded.

Table 1. *Analyses of crude and purified samples of potassium hyaluronate*

Sample	Glucosamine* (%)	N* † (%)	Ash‡ (%)	Moisture (%)	Relative viscosity of 0·1% solution in water (water =1·00)
A	29·2	4·89	20·9	15·4	—
A purified	36·0	3·28	13·1	10·1	—
E	20·9	4·13	27·6	15·4	4·94
E purified	37·2	2·90	18·0	14·0	3·72
Calculated§	41·7	3·21	19·8	—	—

* Corrected for moisture but not for ash content.
† By the micro-Kjeldahl method.
‡ As sulphate.
§ Calculated from the disaccharide consisting of *N*-acetylglucosamine and potassium glucuronate.

The simplified growth media

The simplified medium which seemed most likely to be suitable for the production of hyaluronidase by various species of bacteria was that designed by Bernheimer, Gillman, Hottle & Pappenheimer (1942) for the production of erythrogenic toxin from Lancefield Group A streptococci. Sufficient of a commercial dry preparation of casein acid hydrolysate ('Casamino acids', Difco Laboratories Inc.) was substituted for the laboratory-prepared casein hydrolysate to give the medium the same N concentration as that used by the above authors. Sterile solutions of all the growth factors were added to 9 ml. of the autoclaved amino-acid-salt solution, which contained casein hydrolysate, cystine, tryptophan and salts, to give the concentrations indicated by Bernheimer *et al.* (1942). The solutions of pyridoxin, nicotinic acid, adenine, biotin, thiolacetic acid, magnesium chloride and glucose (the latter acidified with one drop of N-HCl/100 ml.) were sterilized by autoclaving at 115° for 15 min.; those of riboflavin, aneurin and glutamine were Seitz-filtered. The final glucose concentration in the media, except where otherwise stated, was 0·25 g./100 ml. Good growth of strains of Lancefield Group A streptococci and *Cl. welchii* type A, and moderate growth of strains of *Cl. septicum*, was obtained in this medium, which will be called Medium A. In Medium B, for the cultivation of Lancefield Group C streptococci, the uracil concentration was maintained at 10 mg./l., and biotin and adenine were omitted (Rogers, 1944*a*). For staphylococci all the growth factors except aneurin and nicotinic acid (Knight, 1937 *a*, *b*) were omitted (Medium C). The sodium bicarbonate recommended by Bernheimer *et al.* (1942) was not included in any of the media.

RESULTS

Hyaluronidase production by a streptococcus of Group C (*Lancefield*), *Type* 7 (*Griffiths*); *called* C7

The amount of hyaluronidase produced by the streptococcus C 7 was very low when the organism was grown in medium B, despite the luxuriant growth. McClean & Hale (1941) showed that the addition of hyaluronate to a peptone medium increased hyaluronidase production by *Cl. welchii*,

and McClean (1941) obtained similar results for various groups of streptococci. The influence of the inclusion of hyaluronate in the simplified medium was examined as follows:

Three sets of tubes of medium B were inoculated, each tube receiving two drops from the same glucose broth culture grown for 18 hr. at 37°. One set of tubes was supplemented by hyaluronate (0·1 g./100 ml.) added to the amino-acid-salt mixture before autoclaving, and by glucose (0·25 g./100 ml.) added before inoculation; the second set contained no glucose but hyaluronate (0·1 g./100 ml.); the third set contained only glucose (0·25 g./100 ml.).

Table 2 shows the results obtained in this experiment. The addition of glucose and hyaluronate together completely suppressed enzyme formation, whereas hyaluronate, when present as the sole carbohydrate, stimulated hyaluronidase formation.

Table 2. *The influence of the addition of crude potassium hyaluronate on hyaluronidase production by the streptococcus* C 7

(Hyaluronate added to medium B. M.C.P. = 1/enzyme titre in mucin clot-prevention assay (McClean, 1943).)

Glucose concentration (g./100 ml.)	Hyaluronate concentration (g./100 ml.)	M.C.P.	Final pH* of culture
0·25	0	10–50	6·3
0	0·1	1000	6·8
0·25	0·1	<10	5·6

* As determined by indicators.

The relatively low pH induced by the organisms in the medium which contained both glucose and hyaluronate (Table 2) provides a possible explanation for the suppression of hyaluronidase formation in this medium. To test this the following experiment was done:

Three sets of six tubes of medium B containing phenol red (2 mg./100 ml.) were prepared. Two sets contained 0·25 and 0·5 g./100 ml. of glucose and were called media I and II respectively, the third contained potassium hyaluronate (0·1 g./100 ml.) and glucose (0·25 g./100 ml.) (medium III). All the tubes were inoculated with streptococcus C 7 in the usual manner. Two tubes of each set (cultures *e* and *f*) were buffered with Na-β-glycerophosphate (2·5 g./100 ml.) (Rogers, 1944*b*) and two cultures (*c* and *d*) in each set of the three media were maintained at pH 7·4 during growth by the frequent addition of sterile N-NaOH. The third pair of tubes in each set served as unneutralized control cultures (cultures *a* and *b*).

From Table 3 it will be seen that enzyme production in the presence of hyaluronate is the same when growth takes place both in the buffered and the frequently neutralized cultures (*c* and *d*, *e* and *f*); the presence of hyaluronate causes a great increase in the quantity of enzyme produced under these conditions. Results have been obtained which indicate that the pH of the culture is equally important for hyaluronidase production by the other organisms studied. Therefore Na-β-glycerophosphate (2·5 g./100 ml.) was added to all the simplified media (A, B and C) as a routine.

The response of Streptococcus C 7 *to potassium hyaluronate*

Although streptococcus C 7 was selected here for particular study, results have been obtained which indicate that, in part, they can be extended to other organisms, such as group A streptococci and *Cl. welchii*.

Different amounts of a sample of crude potassium hyaluronate were added to tubes of the amino-acid + salts solution before they were autoclaved. After this the necessary growth factors described for medium B were added. Three tubes of medium were used for each concentration level of the polysaccharide. The method of inoculation, of estimation of enzyme yield, and of growth were as already described. The bacterial-N value was corrected for the N contained in the precipitate formed when the hyaluronate + amino-acid + salt solutions were autoclaved.

Fig. 2 shows that enzyme synthesis per unit wt. of bacterial-N is directly proportional to the amount of crude hyaluronate added to the growth medium. This result may be due to an adaptive response of

Table 3. *The influence of glucose and hyaluronate on hyaluronidase production by the streptococcus* C 7

(Simplified medium B with additions of glucose and hyaluronate; frequently neutralized (cultures *c* and *d*) and in medium containing Na-β-glycerophosphate (2·5 g./100 ml.) (cultures *e* and *f*).
M.C.P. = 1/enzyme titre in mucin clot-prevention assay (McClean, 1943).)

	M.C.P. with		
	hyaluronate absent		hyaluronate present (0·1 g./100 ml.)
Growth conditions	Medium I (glucose 0·25 g./100 ml.)	Medium II (glucose 0·5 g./100 ml.)	Medium III (glucose 0·25 g./100 ml.)
Control (cultures *a* and *b*)	150	80	<10, 640*
Frequently neutralized (cultures *c* and *d*)	330	640	10,000
Buffered with glycerophosphate (cultures *e* and *f*)	300	640	10,000

* The enzyme titres for cultures *a* and *b* in medium III are recorded individually and not as a mean because of the large difference between the duplicates.

the streptococci to the presence of hyaluronate. The polysaccharide used, however, was not pure and the increased enzyme formation might have been due to impurities. The ratio: (stimulation of enzyme production (defined in Table 4) caused by a sample of polysaccharide)/(its content of hyaluronate) should be constant if the streptococci are showing a true adaptive response. The hyaluronate content of the polysaccharide samples was estimated from the amount of glucosamine present after acid hydrolysis. Table 4 shows the influence of six samples of hyaluronate upon hyaluronidase production by the streptococcus C 7.

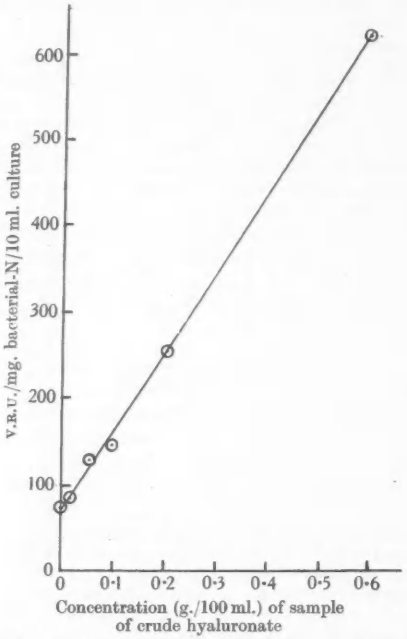

Fig. 2. The influence upon hyaluronidase production by streptococcus C 7 of the concentration of crude potassium hyaluronate in the simplified growth medium. Each point on the curve represents the mean value for three separate cultures. All the points were determined for media inoculated from the same 16 hr. seed culture in glucose broth.

The results for the 'stimulation ratio' (s/w) described above are recorded in column 9 of Table 4. They vary from the mean value of 0·82 by ± 18·8 %. This variation does not differ significantly from ± 12·9 %, the mean value for the difference between the ratio enzyme produced/growth, for the individuals of 25 pairs of duplicate cultures. Thus it is unlikely that the streptococci are stimulated by impurities in the hyaluronate preparations. Moreover, the addition of heparin or of chondroitin sulphuric acid in a concentration of 0·5 g./100 ml. to the simplified medium had no influence upon the enzyme formation. The differences between the glucosamine content of the samples of polysaccharide, however (see Table 4), are of a relatively low order, the greatest being between 20·9 and 37·2 % for sample E before and after purification. It was desirable, therefore, to seek additional evidence on the nature of the increase in enzyme production caused by hyaluronate.

Influence of enzymatically hydrolyzed hyaluronate

If the streptococci are responding to hyaluronate and not to concomitant impurities, hydrolysis by hyaluronidase should decrease or destroy altogether the stimulating properties of the polysaccharides. It was already known that an equimolar mixture of *N*-acetylglucosamine and glucuronic acid had no influence upon enzyme production by the streptococcus C 7 when added to medium B.

Two samples of hyaluronidase were used for hydrolyzing the polysaccharides; one was a purified

Table 4. *The influence of different samples of potassium hyaluronate, added to simplified medium B, on hyaluronidase production by the streptococcus* C 7

(v.r.u. = viscosity reduction units. Different 16 hr. seed cultures of the organism grown in glucose broth were used for inoculation in Exps. I and II.)

Sample of hyaluronate	Analysis of samples: Glucosamine (%)	Analysis of samples: N (%)	Concentration of hyaluronate (g./100 ml. medium)	Exp. no.	v.r.u.	v.r.u./growth*	Stimulation†	s/w‡
A (crude)	29·2	4·89	0·5	I	652	1350	14·2	0·97
B (crude)	24·7	3·32	0·5	I	331	980	10·1	0·83
C (crude)	31·9	2·60	0·5	I	530	1500	16·0	1·01
E (crude)	20·9	4·13	0·25	II	136	408	3·5	0·68
E (purified)	37·2	2·90	0·25	II	163	600	5·7	0·62
None added	—	—	—	I	30·1	88	—	—
None added	—	—	—	II	48·6	131	—	—

* v.r.u./growth = Mean ratio of v.r.u. to mg. bacterial-N/10 ml. culture.

† Stimulation = $\frac{\text{v.r.u./growth on supplemented medium} - \text{v.r.u./growth for unsupplemented medium}}{\text{v.r.u./growth for unsupplemented medium}}$.

‡ Stimulation/wt. hyaluronate as measured by the glucosamine released by acid hydrolysis from the hyaluronate sample.

preparation prepared from testis (bull) by the method of Morgan & McClean (1932). The other enzyme preparation was made from a culture of streptococcus C 7 grown upon a peptone medium and concentrated by precipitation of the culture filtrate with $(NH_4)_2SO_4$, 53 g. being added to 100 ml. of culture filtrate, and subsequent dialysis of the precipitate until free from sulphate ions.

Samples (10 ml. of 0·5 g./100 ml.) of solutions of the K hyaluronate (sample E, purified, Table 1) were mixed with 2·5 ml. of the buffer (pH 7·0) used in the routine viscosimetric assays. Volumes of the enzyme solutions equivalent to 1000 v.r.u. of streptococcal enzyme and 2000 v.r.u. of the testicular preparation were added to the polysaccharide solutions; a few drops of chloroform were added to prevent bacterial growth and the mixtures incubated at 37°. Reducing-sugar determinations were made after 24, 48 and 96 hr.

It was found that, when the streptococcal enzyme was used, hydrolysis ended after about 80 % of the theoretical amount of reducing sugars had been released from the polysaccharide; when the testicular enzyme was used, hydrolysis was only about 66 % complete. The theoretical amount of reducing sugar was calculated on the assumption that hyaluronic acid consists of equimolar proportions of *N*-acetylglucosamine and glucuronic acid (Meyer & Palmer, 1936).

In view of the results obtained when the influence of these hydrolysates upon enzyme production was examined, it seemed important to obtain a more complete breakdown of the hyaluronate by the testicular enzyme. When more of this enzyme was added and the incubation period lengthened by a further 3 days, the degree of hydrolysis was increased to 78 %. Attempts to obtain a further increase, by removing the products of hydrolysis by dialysis or by the addition of more enzyme, failed. The hydrolysates were sterilized and the enzymes inactivated by autoclaving at 115° for 15 min. During this process the hyaluronate solution hydrolyzed by streptococcal enzyme, but not that treated with the testicular enzyme, showed a slight degree of caramelization. It was, however, imperative to inactivate the enzymes used for hydrolysis, since the addition of active hyaluronidase to the test medium would have unduly complicated determinations of enzyme production by the organisms. Hence filtration sterilization could not be used and this small amount of caramelization of hydrolysates prepared with the streptococcal enzymes had to be ignored.

In order to test the influence of the autoclaved hydrolysates upon enzyme synthesis they were mixed with an equal volume of the simplified medium B, prepared at twice its usual concentration.

The medium was also supplemented with an equal volume of undegraded hyaluronate solution (0·5 g./100 ml.) mixed with buffer and sterilized by autoclaving, to serve as a positive control. These media were inoculated in the usual manner with streptococcus C 7 and after 18 hr. the cultures were examined for the quantity of enzyme produced and for mass of growth.

It will be seen (Table 5) that hyaluronate hydrolyzed by the streptococcal enzyme possessed only 25 % of the stimulating properties of the undegraded polysaccharide, whereas the testicular hydrolysates showed undiminished activity.

Since hydrolysis of the hyaluronate by neither of the enzymes was complete, and since the exact nature of the products of enzymic hydrolysis of hyaluronate are unknown, it seemed possible that the testicular enzyme had degraded the polysaccharide predominantly to disaccharide units. If this were so, the remaining linkage between the *N*-acetylglucosamine and the glucuronic acid might be sufficient to stimulate enzyme formation, in which case the materials diffusible through cellophan should also stimulate.

Ten ml. of each of the hydrolysates were dialyzed for 96 hr. against two changes of 50 ml. of distilled water. The two diffusates were combined and concentrated to 10 ml. *in vacuo* (25°); the dialysate was also concentrated to 10 ml. It was found that 80 % of the reducing sugars present in the original hydrolysate had passed through the cellophan. The diffusates and dialysates were sterilized by autoclaving at 115° for 15 min. and tested for their influence upon hyaluronidase formation by the streptococcus C 7 in the usual manner. Table 6 shows that the material which stimulates enzyme formation does not diffuse,

Table 5. *The influence of the products of enzymic hydrolysis of K hyaluronate on hyaluronidase production by the streptococcus* C 7

(The basal growth medium was medium B.)

Exp. no.	Enzyme used for hydrolysis of hyaluronate	Degree of hydrolysis of hyaluronate (%)	Stimulation*	(Stimulation by hydrolysate) / (Stimulation by the unhydrolyzed sample)
1†	Crude streptococcal hyaluronidase	83	0·3	0·26
1*a*†	Purified testicular hyaluronidase	78	3·3	1·03
2	Purified testicular hyaluronidase	65	2·1	1·09
3	Purified testicular hyaluronidase	61	4·7	1·07

* See Table 4.

† Results recorded opposite Exps. 1 and 1*a* were taken from one experiment inoculated from the same seed culture. Therefore, the results under the column headed 'Stimulation' for Exps. 1 and 1*a* can be directly compared.

the diffusates having no influence upon enzyme synthesis.

Table 6. *The influence of fractions from enzymically hydrolyzed hyaluronate on the production of hyaluronidase by streptococcus* C 7

The enzyme used in both experiments was purified testicular hyaluronidase. Basal medium B supplemented by dialysates or diffusates.

Fraction	Stimulation* by whole hydrolysate	Stimulation* by fraction	Stimulation* by maximum amount of undegraded hyaluronate possibly remaining in fraction†
Dialysate	2·1	2·2	0·53
Diffusate		0	—
Dialysate	3·3	1·9	0·21
Diffusate		0	—

* See Table 4.

† The maximum hyaluronate content was calculated by assuming that all the glucosamine released by acid hydrolysis of the dialysates was originally bound in them as undegraded hyaluronate.

The production of hyaluronidase by other organisms

Table 7 shows the quantities of hyaluronidase produced by organisms other than streptococcus C 7, when grown for 16–18 hr. upon the buffered simplified media.

Hyaluronidase production by staphylococci

The lack of response to hyaluronate by the staphylococci may be due to a difference between the mechanism of hyaluronidase synthesis by staphylococci and that used by streptococci and *Cl. welchii*. Or it may be due to the absence from the simplified medium C of factors which limit enzyme formation and thus mask any response to added hyaluronate. The simplified medium C contained only aneurin and nicotinic acid as growth factors. Hyaluronidase production was examined in medium A, which contains a greater range of growth factors, and in the two following empirical media:

(i) Evans's peptone (Evans Sons, Lescher and Webb Ltd.) (3·5 g./100 ml.) sterilized by autoclaving for 15 min. at 115°.

(ii) A laboratory-prepared, Seitz-filtered, papain digest of horse muscle. This medium, when suitably supplemented, was found by Dr W. E. van Heyningen (private communication) to yield culture filtrates from *Cl. welchii* strain S 107 containing a high yield of α-toxin.

Both of these media contained Na-β-glycerophosphate (2·5 g./100 ml.) and glucose (0·25 g./100 ml.). Two sets of each medium were prepared, one of which contained crude K hyaluronate (0·2 g./100 ml.), added to the Evans's peptone medium before sterilization. The viscosity of the papain-digest medium when containing hyaluronate was too great to permit Seitz filtration. Consequently, an autoclaved solution containing hyaluronate (0·5 g./100 ml.) was added aseptically to this medium prepared so that the final N concentration, after dilution with the polysaccharide solution, was 6·1 mg./ml. The media were inoculated in the usual manner.

Table 7. *The production of hyaluronidase by various organisms grown for* 16 *hr. on the simplified medium in the presence and absence of crude hyaluronate*

(M.C.P. = 1/enzyme titre in mucin clot-prevention assay (McClean, 1943); V.R.U. = viscosity reduction units.)

Organisms	Medium	Simplified medium unsupplemented		Simplified medium + K hyaluronate (0·2 g./100 ml.)	
		M.C.P.	V.R.U.	M.C.P.	V.R.U.
Cl. welchii S 107	A	600	83·0	3,200	350
Cl. welchii A 118	A	800	49·8	16,000	496
Streptococcus A 4	A	600	5·5	6,400	159
Streptococcus C 7	B	1600	8·5	24,000	142
Staph. aureus (Wood) 46	C	200	—	100	—
Staph. aureus H	C	1500	115	1,500	133
Cl. septicum K.F.	A	15	2·5	30	4·0
Cl. septicum V.S. 54	A	<5	—	<5	—

The seed cultures of the streptococci and staphylococci were 16 hr. cultures in glucose broth, those for the *Cl. welchii* and *Cl. septicum* strains were 16 hr. cultures in Robertson's heart-meat medium. One drop of the seed culture was used to inoculate 10 ml. of the simplified medium.

Two points of interest will be noted: (i) the absence of response to added hyaluronate by the staphylococci and the strains of *Cl. septicum*; (ii) the very small amount of enzyme formed by the strains of *Cl. septicum*.

Table 8 shows that hyaluronidase formation after 16 hr. growth upon the empirical media which contained hyaluronate was no higher than upon media not containing hyaluronate. At the same time the increased enzyme production by the organisms grown on the papain-digest medium is to be noted.

Table 8. *The production of hyaluronidase by staphylococci*

(Growth for 16 hr. upon simplified and empirical media without and with potassium hyaluronate (0·2 g./100 ml.). All three media contain Na-β-glycerophosphate (2·5 g./100 ml.) and glucose (0·25 g./100 ml.). V.R.U. = viscosity reduction units.)

		No hyaluronate		K hyaluronate (0·2 g./100 ml.)	
Strain	Medium	V.R.U.	V.R.U./growth*	V.R.U.	V.R.U./growth*
Humphrey	Simplified C	138	242	138	216
Farrance	,,	9·7	11·9	10·3	14·7
Wood 46	,,	<5	<25	<5	<25
Humphrey	Evans's peptone	124	165	100	185
Farrance	,,	8·6	15·9	10·3	16·9
Wood 46	,,	12	36	13	—
Humphrey	Papain digest	600	740	510	620
Wood 46	,,	65	170	58·7	133

* V.R.U./growth: see Table 4.

Hyaluronidase production upon simplified medium A was the same as that upon medium B with or without hyaluronate.

Hyaluronidase production by Cl. septicum

Hyaluronidase production by two strains of *Cl. septicum* upon the simplified medium A (Table 4) was very low; it was already known that enzyme production by the strain K.F. was significantly higher upon Evans's peptone medium (30–50 V.R.U.). For this reason the influence upon hyaluronidase formation of the addition of a diffusate prepared from Evans's peptone to medium A was investigated.

The diffusate was prepared by dialyzing a solution of the peptone (60 g./100 ml.), against three changes of distilled water at 0–4°. A biuret test on the third diffusate was only weakly positive. The combined diffusates were evaporated to dryness *in vacuo* at 25°, and the resulting material dissolved in 120 ml. distilled water. This solution contained 22 mg. N/ml.; 60% of the total-N in the peptone had dialyzed through the cellophan. The concentrated diffusate (E.P.D.) was sterilized by autoclaving at 115° for 15 min.

Table 9 shows that the addition of E.P.D. to the medium significantly increased hyaluronidase production by *Cl. septicum*, strain K.F. Enzyme formation was not increased by the inclusion of hyaluronate in either the simplified or the peptone media.

Table 9. *The production of hyaluronidase by* Cl. septicum, *strain K.F.*

Growth in simplified media and peptone media containing glucose (0·25 g./100 ml.) and Na-β-glycerophosphate (2·5 g./100 ml.).

Medium	V.R.U.	V.R.U./growth
Simplified A	2·5	9·5
Simplified A + 0·5 g./100 ml. crude K hyaluronate	4·0	6·1
3·5% Evans's peptone	8·7	28·2
3·5% Evans's peptone + 0·5 g./100 ml. crude K hyaluronate	21·2	36·2
Simplified A	3·2	10·3
Simplified A + 1 ml./100 ml. E.P.D.*	8·8	17·2
Simplified A + 20 ml./100 ml. E.P.D.*	14·6	23·9

* E.P.D. = Evans's peptone diffusate.

DISCUSSION

The one property common to all the organisms so far examined is that optimal formation of hyaluronidase occurs only in well-buffered media; enzyme synthesis apparently ceases when the pH of the culture falls below 6·0. This result may be compared with that obtained by Gale & van Heyningen (1942) with *Cl. welchii* cultivated in a peptone medium, in which hyaluronidase formation per unit volume of culture filtrate was greatest when the initial pH of the culture was 7·5. Larger amounts of hyaluronidase were produced per unit weight of organisms at pH 5·5 than at pH 7·5, however. From the results recorded here the organisms would be unlikely to synthesize appreciable amounts of hyaluronidase in casein hydrolysate media adjusted to pH values as low as 5·5; cultures maintained at a pH other than 7·4 have not been examined.

Comparing the conditions necessary for the formation of hyaluronidase by strains of streptococci, *Cl. welchii* type A, *Cl. septicum* and staphylococci, we can distinguish two groups of organisms: (i) those which form increased amounts of hyaluronidase when hyaluronate is included in the medium; (ii) those which show no such enhanced enzyme production but which require so far unrecognized factors for optimum enzyme formation. Schwabacher, Cunliffe, Williams & Harper (1945) have confirmed the lack of response of staphylococci to hyaluronate added to a broth medium, when a series of freshly isolated strains is used. Thus, just as different organisms have different growth requirements, so also may the requirements (conditions of growth or need for specific substances) for enzyme formation differ with different organisms. *Cl. welchii*, for example, forms no lecithinase (α-toxin) upon simplified media which yield high titres

of hyaluronidase; the presence of other 'toxigenic factors' appears to be necessary.

It appears probable that the enhancement of hyaluronidase production by streptococci and *Cl. welchii* in the presence of hyaluronate is a true adaptation to hyaluronate, and is not due to impurities in the polysaccharide preparations used. The evidence is: (*a*) the ratio of the increase in enzyme production to the amount of pure hyaluronate contained in the crude polysaccharide is constant, within experimental error; (*b*) purification of crude hyaluronate does not decrease its stimulating properties; (*c*) the addition of heparin and chondroitin sulphuric acid to the test medium, had no influence upon hyaluronidase formation.

Dubos (1932) found that an enzyme hydrolyzing the *Pneumococcus* type III polysaccharide was also produced in response to the inclusion of the enzyme substrate in the growth medium used for his soil organism. The only other substance which also stimulated enzyme formation was the aldobionic acid, 4-β-glucosidoglucuronide, formed by acid hydrolysis of the specific polysaccharide. However, the substance responsible for enhancing hyaluronidase production by the streptococcus C7, which is formed when hyaluronate is hydrolyzed by testicular enzyme, will not diffuse through cellophan. Hence it is likely to be of higher molecular weight than a disaccharide. This substance cannot be residual undegraded hyaluronate, since, when a dialysate is used, the final polysaccharide concentration in the test medium cannot be greater than 0·01–0·02 g./100 ml., while the stimulation consistently obtained is that to be expected from four to eight times this concentration. Two explanations of these observations may be suggested. The samples of polysaccharide used may, in fact, be mixtures of two substances of similar composition. If so, the one present in lower concentration, and not hydrolyzed by testicular hyaluronidase, must be responsible for the increased enzyme production. Both of these substances, on this hypothesis, must be considerably hydrolyzed by the streptococcal enzyme. The second possibility is that the difference lies in the degree of hydrolysis brought about by the two enzyme preparations. Very recently Hahne (1945) has stated that crude extracts from swine testis contain two enzymes, only one of which can hydrolyze hyaluronic acid to monosaccharides. It therefore seems possible that hyaluronidase purified from bull testis by the method of Morgan & McClean (1932) consists almost entirely of an enzyme responsible for partial breakdown of the polysaccharide. The streptococcal enzyme preparation, on the other hand, may accomplish a more complete breakdown of hyaluronate. Preliminary observations on the inhibition of streptococcal hyaluronidase by hydrolysates, which were made before the publication of Hahne's (1945) work, may provide tentative evidence for a difference in the degree of breakdown caused by the testicular and streptococcal enzyme preparations. If this hypothesis is substantiated by further work, the stimulation of enzyme formation by the products of hyaluronate hydrolysis by testicular enzyme may be compared with other instances in which the products of partial hydrolysis of a substrate have been shown to stimulate the formation of the corresponding enzyme (Yudkin, 1938).

SUMMARY

1. Peptone-free and protein-free media have been devised in which strains of streptococci, *Cl. welchii* type A, staphylococci and *Cl. septicum* will produce large amounts of hyaluronidase.

2. The conditions controlling enzyme synthesis by these organisms have been studied.

3. All the organisms require an adequately buffered medium.

4. Streptococci and *Cl. welchii* produce hyaluronidase in proportion to the amount of hyaluronate added to the buffered growth medium. This is an adaptive response and is not due to the presence of impurities in the polysaccharide.

5. Hydrolysis of hyaluronate by streptococcal hyaluronidase destroys its stimulating properties, whereas hydrolysis by testicular hyaluronidase does not.

6. Staphylococci and *Cl. septicum* do not respond to the inclusion of hyaluronate either in the simplified or the more complex growth media.

7. *Cl. septicum* and staphylococci require factors present in peptone or digest media for optimal hyaluronidase production. Streptococci and *Cl. welchii* do not require such factors.

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The Linkage of Glutamine in Proteins

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The excess of free amino-groups over the number of lysine molecules in different proteins has been attributed by Chibnall (1942) to the existence of several smaller peptide units within the protein molecule, each of them containing a free terminal α-amino-group. In lactoglobulin these peptide chains are supposed to be held together by non-peptide linkages involving carboxyl and not amino-groups; three possibilities were discussed: (1) ester linkages with the hydroxyamino acids; (2) imide linkages $R.CO.NH.CO.R'$ formed with the amide groups of asparagine or glutamine, and (3) thiol-ester linkages formed with cysteine.

The problem of chain-branching in the protein molecule being under investigation in our laboratory for some years, we have examined the possibility of imide linkages of the type suggested by Chibnall (1942) in formula (1):

$$R_1.NH—OC.CH(NH_2).CH_2.CH_2.CO.NH—OC.R_2. \quad (1)$$

Several proteins were deaminized and then hydrolyzed by boiling with acids. It was expected that the action of nitrous acid on the residue (1) would lead to the formation of α-hydroxyglutaramide residues (2), which after complete hydrolysis should yield free α-hydroxyglutaric acid (3):

$$R_1.NH—OC.CHOH.CH_2.CH_2.CO.NH—OC.R_2, \quad (2)$$

$$R_1.NH_2 + HOOC.CHOH.CH_2.CH_2.COOH + NH_2 + HOOC.R_2. \quad (3)$$

The same acid would be formed if residues of glutamic acid were bound in the peptide chain by their two carboxyl groups, thus leaving the α-amino-group free (formula (4)):

$$R_1.NH—OC.CH(NH_2).CH_2.CH_2.CO—NH.R_2. \quad (4)$$

The hydrolysate of deaminized proteins was tested for α-hydroxyglutaric acid by extraction with ether and oxidation of the ether-soluble fraction with permanganate. Under these conditions a considerable part of hydroxyglutaric acid is oxidized to succinic acid, which can be determined manometrically with succinic dehydrogenase (Krebs, 1937).

If the excess of free amino-groups in the protein molecule were due to mono-amino-acids other than glutamic acid, the corresponding α-hydroxyacids would be formed by the action of nitrous acid, and these would also pass into the ether extract of the hydrolysate.

EXPERIMENTAL

Estimation of α-hydroxyglutaric acid in protein hydrolysates

Pure α-hydroxyglutaric acid was prepared according to Fischer & Moreschi (1912). 2 g. of casein with varying amounts of α-hydroxyglutaric acid, as indicated in Table 1, were boiled with 20 ml. of 23% (w/w) HCl or 8 N-sulphuric acid. The hydrolysate was evaporated under reduced pressure, the residue dissolved in 10 ml. of water and continuously extracted with ether for 8 hr. The ether was removed by distillation, the residue dissolved in 3 ml. of water and filtered. The filtrate and washings, after evaporation to dryness, furnished much less than the expected amount of ether-soluble substances (Table 1), and prolonged extraction with ether did not give higher yields. Hence, it follows that a part of the hydroxyglutaric acid is lost during the boiling with acid. The ether-soluble residue containing α-hydroxyglutaric acid or its lactone (butyrolactone carbonic acid) was dissolved in 5 ml. of 0·1 N-NaOH and the lactone hydrolyzed by keeping the solution in a boiling water-bath for 30 min. The solution was cooled to 25°, acidified with 2 ml. of 2 N-sulphuric acid, mixed with 3 ml. of 0·1 N-permanganate and heated slowly to 60°. Precipitated oxides of manganese were dissolved by the addition of sodium sulphite; then potassium sulphate was added and succinic acid was extracted as described by Goepfert (1940). The ether extract was evaporated, dried, weighed, dissolved in small amounts of water and neutralized by NaOH. Succinic acid was determined in the neutral solution by treatment with succinic dehydrogenase prepared from pigeon's breast muscle (Krebs, 1937). These experiments, as well as those in which α-hydroxyglutaric acid was treated

directly with permanganate, indicate that only 12–23% of the hydroxyglutaric acid is converted to succinic acid (Table 1). Control experiments with pure casein, to which no hydroxyglutaric acid had been added, furnished insignificant amounts of succinic acid (Table 2). There is no doubt, therefore, that the succinic acid found in our experiments (Table 1) originates only from added α-hydroxyglutaric acid.

Table 1. *Yields of succinic acid from α-hydroxyglutaric acid, boiled with casein and hydrochloric acid (in the third experiment with sulphuric acid)*

α-Hydroxyglutaric acid (mg.)	Casein (g.)	Ether-soluble substances: Before oxidation (mg.)	Ether-soluble substances: After oxidation (mg.)	Succinic acid: Amount (mg.)	Succinic acid: Yield (%)
20	2·0	10·2	3·2	2·9	18
20	2·0	—	3·4	3·0	19
20	2·0	12·6	2·0	2·0	12
20	2·0	15·6	7·4	3·0	19
20	0	10·0	3·8	3·8	23
5	2·0	2·0	0·6	0·54	13

Hydrolysis of deaminized proteins

Ovalbumin was prepared from hens' eggs (Kekwick & Cannan, 1936), pseudoglobulin from sheep serum (Haurowitz, Sarafyan & Schwerin, 1941), haemoglobin from horse blood (Zilzer, 1926); in addition, casein (Merck, 'nach Hammarsten') and edestin (Schuchardt) were used. Aqueous solutions containing 5–10% (w/v) of the protein and, if casein or edestin were used, small amounts of NaOH required for the dissolution of these substances, were mixed in a flask with 2 g. of sodium nitrite for 1 g. of protein. The air was displaced by CO_2, the solutions cooled to 1–2° and 50 ml. of N-HCl/g. of protein were added drop by drop. The mixture was kept at room temperature for 12 hr. with occasional shaking and the nitrous gases were then driven out by a current of CO_2. The insoluble deaminized proteins were washed with water. They are intensely yellow substances, which become dark brown when dried at 110°. Their yellow colour indicates that nitro-groups are formed during the deamination.

Dry deaminized protein (0·5–2·0 g.) was boiled with 20 ml. of 23% (w/w) HCl or 8 N-sulphuric acid for 24 hr. When HCl was used, the excess was evaporated *in vacuo* and the residue dissolved in water. The hydrolyzed solutions were extracted continuously with ether, the ether-soluble fraction oxidized, and succinic acid determined as described in the first section. Control experiments were run with the original proteins, without previous deamination.

In some experiments the ether-soluble fraction, extracted from the hydrolysate, was titrated with 0·1 N-NaOH; equivalent weights between 34 and 122 were observed. The microscopical examination of the ether-soluble acids showed a mixture of crystalline and liquid substances. They dissolved easily in water.

The percentage of glutamic acid in pseudoglobulin was determined according to Olcott (1944). 4·0 mg. of hydrolyzed globulin gave 0·495 mg. of amino-N before, and 0·408 mg. of amino-N after, autoclaving.

RESULTS

The experiments recorded in Table 1 demonstrate that 5–20 mg. of α-hydroxyglutaric acid, when mixed with 2 g. of casein and boiled with HCl, furnish 12–24%, on an average 18%, of the theoretical yield of succinic acid. The same method was applied for the approximate estimation of α-hydroxyglutaric acid in hydrolysates of natural and deaminized proteins. Table 2 shows the amount of succinic acid, determined manometrically, and the equivalent amount of α-hydroxyglutaric acid. Since the average yield of succinic acid from α-hydroxyglutaric acid was 18%, the 'corrected' figures of Table 2 have been obtained by multiplying by the factor $100/18 = 5{\cdot}5$. These values are slightly higher for deaminized than for the original proteins. The excess of α-hydroxyglutaric acid, recorded in the last column of Table 2 is negligibly small in comparison with the large amounts of glutamic acid

Table 2. *Estimation of α-hydroxyglutaric acid in the hydrolysates of natural and of deaminized proteins*

(In the first two experiments sulphuric acid was used as hydrolyzing agent, in the other experiments hydrochloric acid.)

Protein	Weight of protein (g.)	Ether-soluble substance: Before oxidation (mg.)	Ether-soluble substance: After oxidation (mg.)	Succinic acid found (mg.)	α-Hydroxyglutaric acid: Equiv. amount (mg.)	α-Hydroxyglutaric acid: Corrected value (mg.)	α-Hydroxyglutaric acid: Excess (mg.)
Casein (native)	2·0	10·0	0·6	0·14	0·18	0·96	—
„ (deaminized)	2·0	9·8	1·5	0·39	0·49	2·7	1·7
Casein (native)	2·0	—	0·8	0·02	0·025	0·14	—
„ (deaminized)	2·0	3·1	1·4	0·17	0·23	1·3	1·2
Globulin (native)	2·0	11·6	4·4	0·13	0·16	0·89	—
„ (deaminized)	2·0	6·3	1·0	0·23	0·29	1·6	0·7
Ovalbumin (native)	2·0	3·1	0·6	0·07	0·09	0·48	—
„ (deaminized)	2·0	5·7	2·1	0·50	0·62	3·4	2·9
Haemoglobin (deaminized)	0·5	—	1·1	0	0	0	0
Edestin (deaminized)	0·5	—	1·8	0·11	0·14	0·75	<0·75

present in the same proteins (Table 3). It corresponds to less than one molecule of hydroxyglutaric acid per protein molecule (Table 3, last column). We conclude, therefore, that *neither glutaminyl residues containing free α-amino-groups (formula* (1)) *nor analogous glutamyl residues* (4) *exist in the examined proteins.*

Table 3. *Molecular ratio of glutamic acid and α-hydroxyglutaric acid per molecule of deaminized protein*

Protein	Assumed molecular weight	Glutamic acid %	Glutamic acid Molecules	Molecules of α-hydroxyglutaric acid per protein molecule
Casein	100,000*	22·0‡	150	0·38
Ovalbumin	45,000†	16·1‡	47	0·44
Globulin	175,000	24·2§	279	0·41
Edestin	50,000‡	20·7‡	71	0·47
Haemoglobin	69,000†	6·75‡	32	0

* Svedberg, Carpenter & Carpenter (1930).
† Svedberg (1934).
‡ Chibnall (1942).
§ Determined in our laboratory by Mrs Vedia Göksu.

Remarks on Table 3. The molecular ratio of α-hydroxyglutaric acid per molecule of deaminized protein has been calculated according to the formula $E \times M/1000W$, where E = excess of hydroxyglutaric acid in mg. (last column of Table 2), M = molecular weight of the protein and W = weight of the analyzed protein sample. The values thus calculated are 'corrected' values; actually only 18% of these quantities have been determined analytically.

The excess of succinic acid obtained from deaminized proteins was observed regularly, although in very small quantities. It might originate from a molecule of glutamic acid or of glutamine bound terminally, by means of its α-carboxyl group, to the long peptide chain forming the protein molecule. But the possibility cannot be excluded that these small amounts of succinic acid arise by the oxidizing action of the previously mentioned nitrocompounds on glutamic acid set free during the hydrolysis of the deaminized proteins.

Our experiments give little support then to the assumption of multiple-free α-amino-groups in the protein molecule. If such groups were present, the hydrolysis of deaminized proteins should furnish a larger amount of ether-soluble α-hydroxyacids. Table 2 indicates, however, that approximately the same quantities of ether-soluble substances were obtained from the deaminized and the original proteins. Although this is contradictory to the assumption of multiple free α-amino-groups in the protein molecule, no definite conclusion can be drawn concerning the existence of these groups, for account must be taken of the fact that some α-hydroxyacids are unstable and might be destroyed by the action of boiling acids.

SUMMARY

1. A method for the approximate estimation of small quantities of α-hydroxyglutaric acid has been described.

2. Casein, ovalbumin, serum globulin, edestin and haemoglobin have been deaminized and then hydrolyzed. Nearly the same amount of ether-soluble substances has been obtained from the hydrolysates of the deaminized and of the original proteins. The ether-soluble fractions contain less than one molecule of α-hydroxyglutaric acid per protein molecule, a fact hardly reconcilable with the assumption of imide linkages, involving glutamine residues within the molecules of the examined proteins.

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Acute Porphyria

SOME PROPERTIES OF PORPHOBILINOGEN

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It was first noticed by Sachs (1931) that the urine of a patient suffering from acute porphyria gave an intense red colour with *p*-dimethylaminobenzaldehyde, in spite of the fact that it did not give Schlesinger's reaction, or the spectrum of urobilin. Moreover, the spectrum of the red dye showed two absorption bands in the green, whereas the product from the reaction of urobilin with *p*-dimethylaminobenzaldehyde gives only one. In addition, the dye was insoluble in chloroform. Exposed to sunlight, the urine darkened and the aldehyde reaction became negative. Waldenström (1937) studied this 'chromogen' in the urine of patients with acute porphyria and failed to isolate it, but demonstrated the darkening of an acid solution of this 'chromogen' with the formation of a red pigment (porphobilin). Waldenström & Vahlquist (1939*a*) mentioned the effect of urea in inhibiting its reaction with *p*-dimethylaminobenzaldehyde and described a method for its estimation in arbitrary units which they used in the study of the amount excreted in cases of acute porphyria (Waldenström & Vahlquist, 1944). These same authors (1939*b*) offered evidence that this colourless chromogen which they called porphobilinogen was a dipyrryl compound and that, in accordance with the theory of Dobriner (Dobriner, Strain & Localio, 1937), two molecules of such a compound could condense to give rise to a porphyrin. Watson & Schwartz (1941) have suggested the value of the insolubility of the condensation product of porphobilinogen and *p*-dimethylaminobenzaldehyde in chloroform as a useful test for the diagnosis of acute porphyria. Porphobilinogen is not excreted in the congenital type of porphyria (Watson, Schwartz & Hawkinson, 1945).

The work here reported was undertaken first to obtain additional information on the reaction between porphobilinogen solutions and *p*-dimethylaminobenzaldehyde, secondly on the interconversion of porphobilinogen and porphobilin, and thirdly on the formation of porphyrin from porphobilinogen. Since porphobilinogen has been put forward as a precursor of the pathological porphyrin in acute porphyria and since the liver is a possible site of porphyrin formation, a search for the presence of porphobilinogen in the liver was thought to be of value.

EXPERIMENTAL AND RESULTS

Preparation of porphobilinogen solution from urine

Several preparations were made from the urine of a case studied (Prunty, 1945) by the method of Waldenström (1937).

In a typical preparation 385 ml. of fresh urine were acidified with 3·5 ml. of acetic acid and, after preliminary filtration, passed through a 10 × 3 cm. column of alumina (Savory & Moore Ltd.). 1% (v/v) acetic acid was then passed through the column until the eluate gave a positive reaction with *p*-dimethylaminobenzaldehyde, when the eluate was collected separately. 250 ml. of pale yellow solution rich in porphobilinogen were obtained. This material was rechromatographed in the same way and a clear colourless liquid obtained still rich in porphobilinogen. The acidity of this solution was brought to pH 6·6 with 10% (w/v) NaOH and the small phosphate precipitate filtered off. The urea content of this solution was only 1 mg./100 ml. The solution was kept on ice.

Preparation of porphobilinogen from liver

Other preparations of porphobilinogen were obtained from liver. 20 g. of liver obtained from the same case at autopsy were finely minced and treated with 50 ml. of 0·9% NaCl containing 1% (v/v) acetic acid and a few drops of toluene added. The suspension was shaken for 1 hr. and then refrigerated overnight. The solid residue was centrifuged down. The supernatant liquor was cleared by the addition of two-fifths of its volume of 20% (w/v) trichloroacetic acid and filtered. The filtrate was passed through an alumina column, a brown band of pigment remaining at the top of the column. The porphobilinogen was eluted by passing 10 ml. of distilled water through the column and the volume made up to 25 ml. It contained 8 units of porphobilinogen (see below), equivalent to 365 units for the whole liver.

The solution was colourless and behaved in a similar manner to that of the porphobilinogen eluates obtained from urine. The absorption bands of the product with *p*-dimethylaminobenzaldehyde were the same, namely, 575–550 and 530–515 mμ, and the solution showed no green fluorescence when tested for urobilinogen according to Schlesinger's technique.

The reaction of porphobilinogen with p-dimethylaminobenzaldehyde

The *p*-dimethylaminobenzaldehyde reagent used was that employed by Waldenström & Vahlquist (1939*a*). A solution of 20 g. *p*-dimethylaminobenz-

aldehyde in 500 ml. of 36 % (w/w) HCl was diluted with 500 ml. of distilled water. Equal volumes of porphobilinogen solution and the reagent were mixed and allowed to stand at room temperature for 80 sec. The intensity of the colour was measured with a Hilger absorptiometer using Ilford filter 605. 1 unit/ml. was taken as $E^{550°}_{1\,cm.} = 0{\cdot}545$ (Waldenström & Vahlquist, 1939*a*).

Fig. 1 shows that the light absorption of the product of the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde slightly deviates from Beer's law.

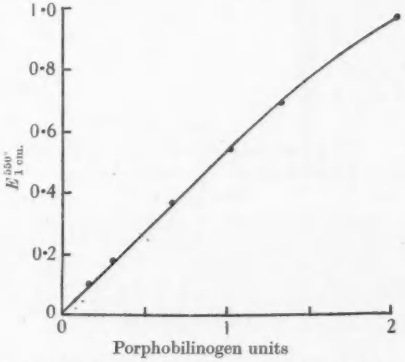

Fig. 1. Calibration curve for the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde.

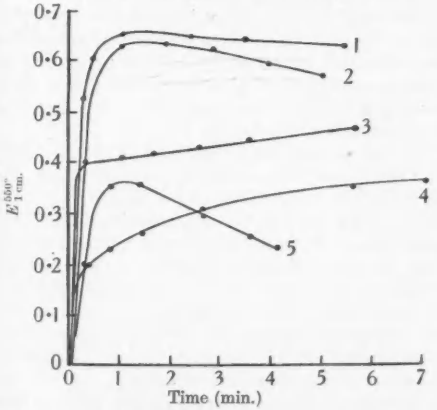

Fig. 2*a*. Effect of inhibiting substances on the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde. 1. Porphobilinogen from urine partially purified by chromatography. 2. Urine from acute porphyria diluted to 1/17 (urea concentration 0·02 M). 3. Porphobilinogen solution (1) plus two-fifths of the volume of normal urine (urea concentration 0·07 M). 4. The same porphobilinogen solution containing 0·33 M-urea. 5. Porphobilinogen solution prepared by chromatography from liver of acute porphyria.

Influence of interfering substances on the course of the reaction

The rate of reaction of solutions of porphobilinogen with *p*-dimethylaminobenzaldehyde at room temperature is shown in Fig. 2*a*. It will be seen that urea both depresses the rate of reaction and

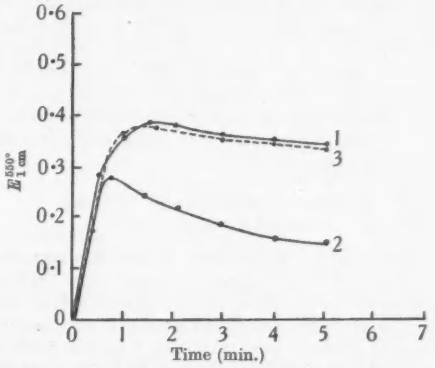

Fig. 2*b*. 1. Porphobilinogen solution after chromatography. 2. The same solution containing 0·01 M-ascorbic acid. 3. The same solution containing 0·01 M-oxidized ascorbic acid.

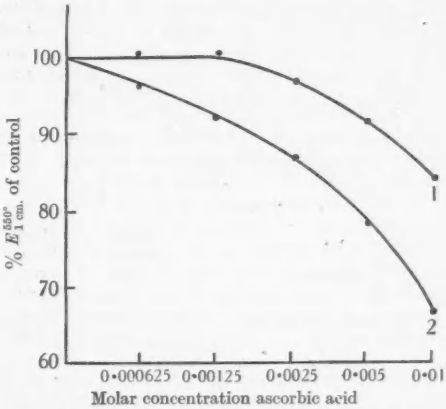

Fig. 3. Ascorbic acid concentration and the inhibition of the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde. 1. 50 sec. after the addition of *p*-dimethylaminobenzaldehyde. 2. 90 sec. after the addition of the reagent.

inhibits the complete formation of the colour. This is also demonstrated after the addition of urine. Creatine and creatinine were found to have no such effect.

The solution of porphobilinogen obtained from the liver clearly contains some substance accelerating the rate of fading, but this was not identified.

Ascorbic acid (Fig. 2*b*) also has a marked effect on inhibition of the reaction and acceleration of fading. An equivalent amount of ascorbic acid which had been quantitatively oxidized by iodine is without effect (Fig. 2*b*, curve 3). The relation of inhibition to concentration of ascorbic acid is given in Fig. 3.

Hydrogen peroxide causes almost complete inhibition of the reaction. The addition of hydrogen peroxide to a solution of *p*-dimethylaminobenzaldehyde gives an unstable light blue product. 10 ml. of H_2O_2 (20 vol.) added to 5 ml. of porphobilinogen solution, and the whole diluted to 25 ml., gave a very transitory red coloration, rapidly turning to green.

The effect of reduction with Terwen's solution on the reaction

The reduction of urobilin to urobilinogen can be effected by means of a suspension of ferrous hydroxide in alkaline solution (Terwen, 1925). The effect of this method of reduction on the urinary pigments of acute porphyria was therefore investigated. Fresh specimens of urine so treated gave a more intense reaction with *p*-dimethylaminobenzaldehyde, but the effect was only small, and no appreciable reduction of the amount of pigment in the urine was apparent. On the other hand, if the urine had been allowed to stand for 24 hr. before treatment with the reducing agent, there was actually a decrease in the intensity of the reaction with *p*-dimethylaminobenzaldehyde (Table 1).

Table 1. *The effect of Terwen's reduction on porphobilinogen reaction of urine*

		$E^{550°}_{1\,cm.}$ Before reduction	$E^{550°}_{1\,cm.}$ After reduction
Fresh urine diluted to 1/50	(*a*)	0·385	0·401
	(*b*)	0·167	0·180
Urine after 24 hr. standing diluted to 1/50	(*c*)	0·261	0·200
	(*d*)	0·340	0·305

The effect of heating with alkali on the course of the reaction of porphobilinogen with p-*dimethylaminobenzaldehyde*

Fig. 4 shows the results obtained on: (i) a specimen of urine diluted to 1/25; (ii) the same urine reduced with Terwen's reagent and diluted to 1/25; (iii) the filtrate after the reduction, containing 0·06 M-NaOH, heated 15 min. in a boiling water-bath and diluted to a final dilution of 1/25. Heating with alkali causes a slight reduction in the colour intensity after reaction with *p*-dimethylaminobenzaldehyde, but a marked change in the absorption spectrum of the product. Solution (ii) showed the typical spectrum produced with porphobilinogen (575–550 and 530–515 mμ), but (iii) showed the band at 575–550 mμ persisting with the disappearance of the band at 530–515 mμ.

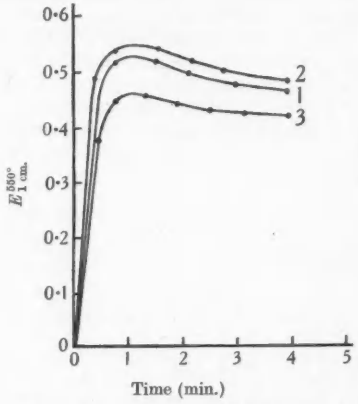

Fig. 4. Effect of treatment of porphobilinogen with alkali on its reaction with *p*-dimethylaminobenzaldehyde. 1. Urine of acute porphyria diluted to 1/25. 2. The same urine after treatment with Terwen's reagent. 3. The same material as (2) after heating 15 min. in the boiling water-bath with 0·06 M-NaOH.

Conversion of porphobilinogen to porphobilin

Relation to pH *of solution.* This experiment was carried out in a 0·5 M-phosphate, 0·25 M-citric acid buffer. To 100 ml. of a porphobilinogen solution were added 43·25 ml. of N-NaOH to neutralize the solution, giving 0·302 M-acetate. The small phosphate precipitate which carried down a trace of porphobilin was filtered off.

To 5 ml. of this filtrate were added 2·5 ml. of 36 % (w/w) HCl and the mixture diluted to 25 ml. For the other pH values 5 ml. amounts were added to 10 ml. buffer solution and diluted to 25 ml. 3 ml. quantities of these solutions were (*a*) treated with 3 ml. *p*-dimethylaminobenzaldehyde reagent and (*b*) heated 10 min. in a boiling water-bath and, after cooling, treated with the reagent. At the same time the absorption density of the solution was determined before and after heating, using Ilford filter 603 (maximum absorption at 490–500 mμ). In this way comparative data for the porphobilin content were obtained. Almost complete separation of the porphobilinogen-*p*-dimethylaminobenzaldehyde compound and porphobilin was possible with the two Ilford filters employed (605 and 603 respectively). Fig. 5 shows the relation of porphobilinogen loss to pigment production, 100 % pigment formation being taken arbitrarily as the amount of pigment found in the most acid solution. The control curve 3 shows that a small part of this is due to humin. The

solutions after heating showed the typical band of porphobilin at 500mμ.

Fig. 6 indicates the rate of formation of porphobilin at the optimum pH of 5·2.

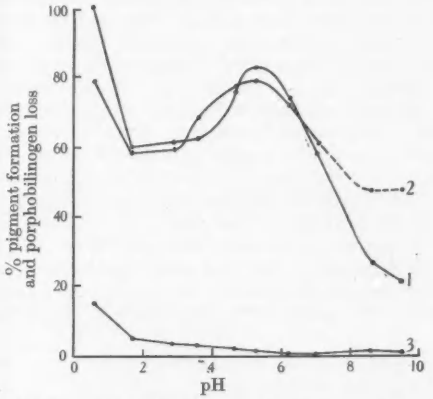

Fig. 5. The relation of pH of a porphobilinogen solution to the formation of porphobilin. Heated 10 min. in a boiling water-bath. 1. Porphobilin produced. 2. Porphobilinogen loss. 3. Pigment formation in a specimen of normal urine (control).

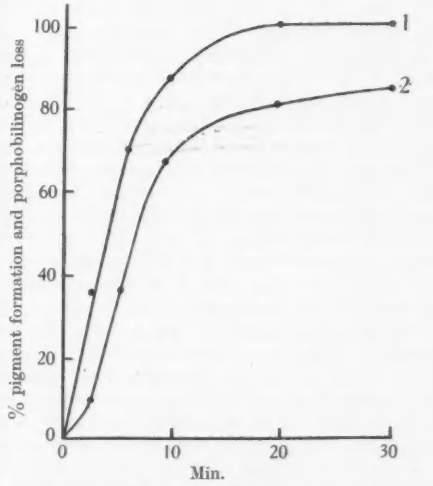

Fig. 6. The rate of formation of porphobilin at the optimum pH (5·2). Porphobilinogen solution heated in a boiling water-bath. 1. Porphobilin produced. 2. Porphobilinogen loss.

Effect of ascorbic acid and hydrogen peroxide. 5 ml. amounts of porphobilinogen solution were diluted with 10 ml. buffer (pH 5·2) and volumes made up to 25 ml. The solutions were heated 5 min. in a boiling water-bath, except where otherwise stated. The data in Table 2 show the effect of ascorbic acid and hydrogen peroxide on the pigment production. Owing to the interfering action of both ascorbic acid and hydrogen peroxide in the *p*-dimethylaminobenzaldehyde reaction with porphobilinogen, no quantitative assessment of the porphobilinogen loss under these conditions was made.

Table 2. *Conversion of porphobilinogen to porphobilin*

(Solutions were heated for 5 min. at 100°.)

	Increase in density with Ilford filter 603 (% of density after 30 min. heating)
Control solution (pH 5·2)	73
Control solution with 5 ml. normal urine	78
Control solution with 50 mg. ascorbic acid at pH 5·2	60
Control solution with 10 ml. H_2O_2 (20 vol.)	Nil
Do., stood 30 min. at room temperature in daylight	Nil
Do., 60 min.	Nil

Porphyrin formation from porphobilinogen

In vitro *experiment.* In one experiment two quantities of 5 ml. of a porphobilinogen solution containing 1·5 units/ml. were buffered to pH 5. In one, the control, 1 ml. conc. HCl was added to adjust acidity to pH 1 and the solution immediately treated with 1 g. Keiselguhr and allowed to stand for 2 min. The suspension was then dealt with as described by Rimington (1943*b*). The fluorescence intensity of the final product in 0·1 N-NaOH was determined by means of a Hilger fluorimeter with a Chance no. 3 filter in the secondary beam. The fluorescence was not the red of a porphyria but largely greenish in colour, the intensity of which gave a control reading equivalent to 21 μg. of uroporphyrin in the solution.

The second quantity was heated in a boiling water-bath under an air condenser for 30 min., by which time much porphobilin had been formed. After cooling it was treated in the same manner as the control. The fluorescence now obtained was equivalent to 71 μg. of uroporphyrin, but it was not quite a pure red, being contaminated with the greenish fluorescence of the control. The difference observed between the heated solution and the control indicated therefore a formation of 50 μg. of porphyrin, and the solution showed very weak absorption bands at approximately 570 and 535mμ. Acidification with acetic acid and ether extraction gave no detectable coproporphyrin in the ether extract.

In vivo *experiment*. A healthy adult male rabbit was maintained on the laboratory stock diet in a metabolism cage for a 5-day control period. Urine and faeces were collected. The rabbit was then given 20 ml. of a porphobilinogen solution intraperitoneally for a 3-day period. This solution was prepared as outlined above and after neutralization to pH 6·6 with 10 % NaOH, it was Seitz-filtered and kept in the refrigerator. The porphobilinogen content was 2 units/ml. On account of the observation that treatment of a patient suffering from acute porphyria with methionine suggested that this amino-acid might possibly enhance porphyrin production (Prunty, 1945), the rabbit was given, for a final 3-day period, 20 ml. of this solution and in addition 0·5 g. of methionine intraperitoneally.

The porphyrin excretion in urine was determined by the method of Rimington (1943 *a*, *b*) and in the faeces by that previously described (Prunty, 1945).

Table 3. *Coproporphyrin excretion in male rabbit receiving porphobilinogen*

Period	Urine (ml./3 days)	Faeces (g./3 days)	Coproporphyrin (μg./3 days) Urine	Coproporphyrin (μg./3 days) Faeces	Total coproporphyrin (μg./3 days)
I. Control	116	69	8·8	33	41·8
II. Porphobilinogen injected	145	51·5	9·8	21	30·8
III. Porphobilinogen and methionine injected	159	27	7·5	18	25·5

Table 3 records the results obtained. During the experimental period the appetite of the rabbit diminished, but there were no other untoward effects. This probably explains the drop in the faecal output, and in turn the diminishing coproporphyrin excretion. The urine analysis and faecal chromatograms afforded no evidence of the presence of uroporphyrin, in spite of the fact that observation of such chromatograms in ultra-violet light is a very sensitive test for porphyrin. The urine during the periods of porphobilinogen injection contained small amounts of porphobilinogen identified spectroscopically after reaction with *p*-dimethylaminobenzaldehyde, but the amounts were too small to make estimation practicable in the concentrated urine. At the conclusion of the experiment the animal was sacrificed, but showed no changes on post-mortem examination.

DISCUSSION

Isolation of porphobilinogen has not, so far, been possible, owing largely to its complete insolubility in organic solvents (Waldenström, 1937). The results show that the present method of determining relative amounts of porphobilinogen in urine is only approximate on account of the interfering activity of substances present in the urine. The results of Waldenström & Vahlquist (1939*a*) with urea have been confirmed and extended and the effect of ascorbic acid studied in some detail. Preliminary dilution of the urine, as advocated by these authors, reduces the effect of such interfering substances to a minimum.

Waldenström & Vahlquist (1944) have shown that the colour of the urine of patients with acute porphyria depends upon its pH, and Waldenström (1937) found that porphobilinogen was transformed to porphobilin in acid solution, or in the presence of hydrogen peroxide in daylight. Fig. 5 shows the effect of acidity on the amount of porphobilin produced in partially purified solution of porphobilinogen, and indicates that there is an optimum at pH 5·2. There is a parallel between the amount of pigment formed and the loss of porphobilinogen from the solution in the acidic medium. In the alkaline medium, the porphobilinogen breakdown continues in the absence of pigment formation. Heating a solution of porphobilinogen with alkali reduced the intensity of the colour produced with *p*-dimethylaminobenzaldehyde, and was accompanied by the disappearance of the weaker of the two spectral bands shown by the porphobilinogen-*p*-dimethylaminobenzaldehyde compound, thus indicating the formation of some new product. This fact has also been observed by Waldenström & Vahlquist (1939*b*). The results indicate that the reaction porphobilinogen-porphobilin is not reversible under the conditions investigated. In addition it has been found that simple oxidation of porphobilinogen with hydrogen peroxide, at the pH which is optimal for the formation of porphobilin in the presence of air, does not lead to pigment production, although ascorbic acid seems to be slightly effective in inhibiting pigment formation. Terwen's solution did not lead to significant reduction of porphobilin.

Attempts to convert porphobilinogen to porphyrin have only partially confirmed Waldenström's findings. There was evidence of production of uroporphyrin on heating at pH 5, but no evidence of porphyrin formation when porphobilinogen was injected into the rabbit. Waldenström & Wendt (1939) were successful in obtaining uroporphyrin by similar means. In a case of acute porphyria studied by the author (Prunty, 1945), some evidence was obtained which suggested the possibility that methionine administration favoured the production of uroporphyrin, but the experimental evidence in

the rabbit, to investigate this point, was negative. That porphobilinogen has here been demonstrated to be present in the liver in acute porphyria and that uroporphyrin has been obtained from liver and from faeces (Grinstein, Schwartz & Watson, 1945; Prunty, 1945), is of considerable significance. These facts do not accord with the view put forward by Waldenström & Vahlquist (1944) that the porphyrin formation in this condition is purely a secondary event, taking place after the formation of the urine.

SUMMARY

1. The preparation of purified solutions of porphobilinogen from the urine and from the liver of a patient suffering from acute porphyria is described.

2. The reaction of these solutions with *p*-dimethylaminobenzaldehyde is studied. Urea and ascorbic acid inhibit the reaction. Creatine and creatinine are without effect.

3. The formation of porphyrin from porphobilinogen heated in acid solution is confirmed. Quantitative data afford no evidence for formation of porphyrin from porphobilinogen injected into the rabbit.

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Studies on the Metabolism of Semen

1. GENERAL ASPECTS. OCCURRENCE AND DISTRIBUTION OF CYTOCHROME, CERTAIN ENZYMES AND COENZYMES

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Semen as ejaculated is composed of two parts, the spermatozoa or 'sperm' and the seminal fluid or 'plasma'. There are numerous analytical data on the chemical composition and the metabolic activities of semen, but for the most part they refer to whole semen without drawing a clear distinction between the sperm and the plasma (for references see Anderson, 1945). Yet, from many points of view, the two components of the semen must be considered separately, in the same sense as, for instance, the blood cells and blood plasma.

The spermatozoa exhibit two outstanding biological properties, fertility and motility, and they possess a characteristic metabolism of their own, maintained by a number of intracellular catalysts. In the presence of oxygen they show a considerable respiratory activity which is correlated significantly with both the number of cells and the intensity of movement; at the same time, the rate of respiration gives a quantitative measure of the viability of spermatozoa and is therefore used by some investigators as an indicator in assessing the potential fertilizing capacity of the semen (Walton, 1939). In the absence of oxygen, the activity of spermatozoa is maintained by glycolytic processes. Recent research indicates that the glycolysis, like the respiration, is correlated with the motility and fertility of spermatozoa. There seems to be little doubt that in whole semen the spermatozoa utilize glucose as the substrate for glycolysis (cf. MacLeod, 1943*a*). This is supplied by the plasma which in itself is devoid of a glycolyzing system. It is frequently said that the seminal plasma acts towards the spermatozoa both as a fluid vehicle for their transport and as a

nutrient medium. However, the significance of the seminal plasma goes far beyond that description. It is not a homogeneous fluid but a complex mixture secreted by several important glands. It carries a large number of highly active enzymes and it has a metabolism of its own.

This paper deals with the presence and distribution in the semen of several constituents. First, it will be shown that the spermatozoa contain a complete cytochrome system. Next, evidence will be given for the existence in the spermatozoa of adenosinetriphosphate (ATP). It will be shown that during the metabolic activity of spermatozoa, ATP undergoes characteristic changes which suggest that the function of this coenzyme is in some way connected with the motility of the cells. A study is also included of seminal deaminases and phosphatases with special reference to those enzymes which act on ATP and on other adenyl derivatives (preliminary communication, Mann, 1945*a*).

EXPERIMENTAL

Methods

The ejaculated semen of the ram, bull and boar was used. The semen of rams, being composed of nearly equal parts of sperm and plasma, has a higher concentration of spermatozoa (2·5–5 million/μl. semen) than that of other mammals and is therefore particularly suitable for metabolic studies on spermatozoa. Usually a mixed sample of semen was used, collected from seven rams, the volume varying from 5 to 18 ml. according to the season. Bull semen (0·8–1·1 million cells/μl. semen) lends itself to studies on spermatozoa as well as on the seminal plasma; single ejaculates were used varying in volume from 5 to 6·5 ml. Boar semen, although very voluminous (250 ml. in a single ejaculate), has but a small proportion of spermatozoa. Ram and bull semen was collected by Dr A. Walton, and that of boar by Miss C. Williams-Ellis, at the Animal Research Station, Cambridge. The method of collection was that described by Walton (1945). It was brought to the laboratory within 1 hr. after ejaculation. The sperm was separated from the seminal plasma by centrifugation. In order to remove the plasma from the spermatozoa the samples were suspended in several volumes of a calcium-free Ringer solution and centrifuged. Finally, the washed cells were resuspended in fresh Ringer solution up to the original volume of the whole semen, thus yielding a suspension of spermatozoa equal in concentration to that of the original whole semen. In a few experiments, e.g. in spectroscopic experiments on bull spermatozoa, the volume of suspensions was kept smaller than that of the original semen. The suspensions of washed spermatozoa are referred to as the 'washed sperm', and the results of chemical estimations carried out with the washed sperm are expressed in mg./100 ml. washed sperm, equivalent to 100 ml. semen.

The washing of the spermatozoa usually causes only little damage to the cells provided that certain precautions are observed. Considerable damage can easily result, for instance, from vigorous shaking of the sperm suspension. To avoid this type of inactivation it is convenient to add the Ringer solution gradually to the centrifuged sperm in the centrifuge tube and to stir it up gently with the bottom-end of a loosely fitting test-tube till a homogeneous suspension is obtained. As a rule, the washing of spermatozoa should not be repeated more than once. Exhaustive washing, particularly if carried out with a large volume of Ringer solution, may cause a great deal of damage. Only in exceptional cases such as, for instance, in studies on phosphatases and diastases, is it necessary to wash the spermatozoa more than once so as to achieve a complete separation of the enzymes present in the spermatozoa from those carried by the seminal plasma. Another factor which may influence the experimental results concerns the time and speed of centrifugation; these should be kept as low as possible. In this respect, ram spermatozoa seem to be much more resistant and therefore much more suited for metabolic studies than bull or boar spermatozoa. Less time is required to achieve a separation by centrifugation of the sperm from the plasma in the case of ram than that of other animals.

The observations on cytochrome were made by means of a microspectroscope ocular attached to a microscope; the tube containing the sperm was illuminated by a strong Pointolite lamp. Cu was determined in the semen as diethyl-dithiocarbamate, Zn with dithizone and Fe with $\alpha\alpha'$-dipyridyl. The production of lactic acid was followed by the method of Friedemann, Cotonio & Shaffer (1929), using the apparatus of Lieb & Zacherl (1932); before the estimation of lactic acid, protein was removed with trichloroacetic acid and the extracts treated with copper-lime. Ammonia was estimated according to Parnas & Heller (1924). The acid-soluble phosphate was estimated in trichloroacetic acid extracts by the method of Lohmann & Jendrassik (1926). The following fractions were determined: (1) $P_{inorg.}$, the true inorganic phosphate determined as $MgNH_4PO_4$; (2) P_0, phosphorus determined as phosphate which reacts directly with ammonium molybdate; (3) P_7, P_{30} and P_{180}, the phosphorus which appears as orthophosphate after 7, 30 and 180 min. hydrolysis at 100° with N-HCl; (4) $P_{tot.}$, phosphorus as the total acid-soluble phosphate after incineration. The alkali-hydrolyzable phosphate of phosphotrioses was determined by the method of Meyerhof & Lohmann (1934).

Cytochrome

For the spectroscopic examination, the washed sperm of ram and bull was used. 2 ml. of a thick suspension of spermatozoa (3×10^9 cells/ml.) were placed in a Thunberg tube and examined under the microspectroscope, the thickness of the layer being increased gradually from about 0·2 to 1 cm. In a freshly prepared suspension the cytochrome is only partially reduced and the absorption bands are therefore weak. On standing, however, or better still on adding a little glucose and replacing the air by nitrogen, the cytochrome becomes fully reduced and the suspension then shows distinctly the spectrum of all three reduced cytochromes, *a*, *b* and *c*. The absorption band of the cytochrome *a* is particularly strong and, on passing CO through the sperm, the formation of the CO-compound can be clearly observed. This compound is identical with the CO-compound of cytochrome oxidase or cytochrome a_3

as first described in heart-muscle preparations by Keilin & Hartree (1939). If a suspension of spermatozoa is vigorously shaken in air, the cytochrome is oxidized and the bands disappear. In the presence of cyanide, however, the oxidation is inhibited and cytochrome remains in the reduced form. No appreciable change in the spectrum of cytochrome was observed in ram and bull spermatozoa after they had been preserved for 1 week at 10°, under anaerobic conditions. The complete spectrum of cytochrome was also observed in whole semen of ram and bull. However, in the case of bull semen it was necessary to carry out the examination of a layer about 1 cm. thick, which is quite understandable in view of the comparatively low density of spermatozoa. No cytochrome was found in the seminal fluid from which spermatozoa had been removed.

Fe, Zn *and* Cu

One large sample (15 ml.) of mixed ram semen was examined for its content of Fe, Zn and Cu. The three elements were determined separately in the washed spermatozoa and in the seminal plasma. Table 1 shows that the concentration of all three elements is higher in the spermatozoa than in the plasma, and this in spite of the fact that the sperm had been repeatedly washed with Ringer solution. The form in which the three elements occur remains to be investigated.

Table 1. *Distribution of* Fe, Zn *and* Cu *in ram semen*

	100 ml. semen contain	
	In sperm	In plasma
Fe (mg.)	0·68	0·16
Zn (mg.)	0·70	0·28
Cu (mg.)	0·12	0·05

Acid-soluble phosphorus

The difference in the distribution of elements between the sperm and the plasma is also noticeable with P. In the ram semen, the readily hydrolyzable P compounds are mainly concentrated in the spermatozoa. Table 2 shows the distribution of the acid-soluble P in freshly washed sperm. On standing, the inorganic phosphate increases, mainly although not exclusively at the expense of the readily hydrolyzable P fraction.

Table 2. *Acid-soluble phosphorus in ram spermatozoa*

(4×10^9 cells/ml.)

($P_{inorg.}$ = true inorganic phosphate determined as $MgNH_4PO_4$; P_0 = phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_7 and P_{30} = phosphorus which appears as orthophosphate after 7 and 30 min. hydrolysis at 100° with N-HCl; $P_{tot.}$ = phosphorus as the total phosphate after incineration of the trichloroacetic acid extract.)

$P_{inorg.}$	P_0	P_7	P_{30}	$P_{tot.}$
5·1	5·9	9·8	10·5	39·0 mg. P/100 ml.

Adenosinetriphosphate

As shown in Table 2, a large proportion of the acid-soluble P present in the ram sperm yields orthophosphate after 7 min. hydrolysis with N-HCl, and thus behaves like a typical readily hydrolyzable P compound. Lardy, Hansen & Phillips (1945) observed the occurrence of a similar P fraction in bull sperm and suggested that it might be adenosinetriphosphate. However, the possibility had to be considered that the substance might be one of the many adenosine polyphosphates other than ATP or one of the inorganic polyphosphates such as occur, for example, in moulds and yeast (Mann, 1944). In order to obtain more definite information with regard to the chemical nature of the readily hydrolyzable P compound, an attempt was made to isolate it from the spermatozoa. The procedure was based on the method recommended by Parnas & Lutwak-Mann (1935) for the quantitative estimation of adenosinetriphosphate in skeletal muscle.

10 ml. fresh ram semen were used, containing 3×10^9 cells/ml. It was diluted with 15 ml. Ringer solution, centrifuged, and the two parts, the sperm and the dilute plasma, were brought with Ringer solution to 20 ml., cooled in ice and twice extracted with 10 ml. 10% cold trichloroacetic acid, centrifuging each time. Tests carried out on small samples of the extracts have shown that most of the acid-soluble P passed into the first extract. The combined extracts were precipitated with Ba acetate at pH 8 and centrifuged 30 min. later. The supernatant solutions gave no further precipitate with Ba acetate and were discarded; they contained no readily hydrolyzable P but showed the presence of difficultly hydrolyzable P compounds.

The Ba precipitates were washed with dilute Ba acetate and dissolved in 5 ml. 0·05 N-HCl. Ba was then removed by adding Na_2SO_4, the $BaSO_4$ precipitate spun down and washed twice with 4 ml. 0·05 N-HCl. The washings were added to the main solution, neutralized, with bromo-thymol blue as indicator, and made up to 15 ml. This solution was divided into three equal parts, *A*, *B* and *C*.

A was incubated for 2 hr. at 37° with 1 ml. frog muscle pulp, which serves as a source of adenyl deaminase. The incubation was terminated by the addition of 10 ml. saturated borax, and NH_3 was estimated in the Parnas and Heller apparatus.

B was treated with 10 ml. saturated borax; to this was added 1 ml. muscle pulp which had been incubated for 2 hr. at 37° and NH_3 was estimated. The difference between the NH_3 content in fractions *A* and *B* represents the NH_3 liberated by the enzymic deamination and corresponds to the NH_2 of ATP.

C was used for the determination of P_0 and P_7. The results of analysis were as follows:

Spermatozoa. *A*: 0·029 mg. NH_3-N. *B*: 0·006 mg. NH_3-N. Deaminated enzymically 0·023 mg. NH_3-N or 1·64 μmol.

C: 0·112 mg. (P_7–P_0) or 3·60 μmol. P; molecular ratio NH_2-N : P = 1 : 2·2; ATP content in spermatozoa from 10 ml. semen (3×10^9 cells/ml. semen): 0·069 mg. NH_2-N and 0·336 mg. readily hydrolyzable P.

Seminal plasma. *A*: 0·009 mg. NH_3-N; *B*: 0·006 mg. NH_3-N; deaminated enzymically 0·003 mg. NH_2-N; *C*: 0 mg. (P_7–P_0).

The analytical data show that the P compound present in spermatozoa has the properties of adenosinetriphosphate; it forms the Ba salt under identical conditions, and the molecular ratio between NH_3 liberated by the muscle deaminase and P liberated by the acid hydrolysis agrees satisfactorily with the theoretical ratio 1 : 2 required for ATP. Calculated from these figures, the amount of ATP contained in the spermatozoa of 100 ml. ram semen (3×10^9 cells/ml. semen) is 0·69 mg. ATP-amino-N and 3·36 mg. ATP-readily hydrolyzable P. It appears that in the semen all the ATP is confined to the cells.

The content of ATP was determined five times, using mixed samples of semen from the same seven rams, and once in bull semen (6·5 ml.). In each case the compound was first separated in the form of its Ba salt and then NH_2-N determined as NH_3. The estimation of the readily hydrolyzable P was sometimes omitted, since it was found to be less accurate; moreover, the P analysis does not always provide a reliable criterion for assessing the ATP content, especially under conditions where the occurrence of other readily hydrolyzable P compounds cannot be excluded with certainty. In the case of ram semen, the lowest content of ATP-amino-N recorded was 0·5 mg./100 ml., and the highest 1·5 mg./100 ml. The highest content of ATP in spermatozoa was observed in December, coinciding with the peak of the breeding season, and the lowest in April, at the end of the season. However, as this observation was made during one season only, it requires repetition for full confirmation. The ATP-amino-N of bull semen was 0·41 mg./100 ml., a rather high value considering the relatively low concentration of spermatozoa in the bull semen.

Behaviour of ATP in spermatozoa. For these experiments the ram sperm was used after the removal of the plasma by washing.

In one experiment two equal samples were incubated for 2 hr. at 37°, one aerobically, the other anaerobically; the contents of ATP-amino-N were 0·208 and 0·056 mg./100 ml. respectively, showing that the level of ATP in washed spermatozoa is maintained better in air than in nitrogen. In another experiment a sperm suspension was buffered by the addition of 0·05 M-phosphate, pH 7·3, and divided into two equal parts; 0·03 M-glucose was added to one part only, and both were incubated in N_2 for 2 hr. at 37°. Afterwards, the samples were deproteinized with trichloroacetic acid and in the filtrates ATP and lactic acid were determined. The results were 0·55 mg. ATP-amino-N/100 ml. and 605 mg. lactic acid/100 ml. in the sperm incubated with glucose, and 0·15 mg. ATP-amino-N/100 ml. and 35 mg. lactic acid/100 ml. in the sperm incubated without glucose. These results indicate that under anaerobic conditions the ATP content is preserved better in the presence than in the absence of glucose.

Adenosine derivatives other than ATP

Spermatozoa contain some other adenosine derivatives apart from ATP. In order to determine the joint content of these compounds, a method has been worked out similar to that used by Ostern, Baranowski & Terszakoweć (1938) for the estimation of adenylic acid + adenosine-polyphosphoric acids in yeast.

Whole semen, washed spermatozoa and the seminal plasma were each extracted with trichloroacetic acid. The centrifuged and filtered extracts were freed from trichloroacetic acid by ether extraction, neutralized to phenolphthalein by the addition of Na_2CO_3, free NH_3 was removed in the Parnas-Heller apparatus, and the NH_3-free solution neutralized to pH 7. The neutral solutions were then incubated for 2 hr. at 37° with 2 ml. deaminase prepared by grinding 50 mg. rat heart muscle in 0·1 M-phosphate, pH 7. Under these conditions both adenosine and adenylic acid as well as adenosine-polyphosphates are deaminated. The total content was 3·9 mg. NH_2-N/100 ml. whole semen, of which 3·45 mg. were derived from the sperm and 0·45 mg. from the plasma.

Ammonia formation in semen

The total NH_2-N content of the semen as assessed by means of the heart muscle adenyl deaminase undergoes little change during the storage of the semen in spite of the fact that there is a decrease in ATP-amino-N. For example, at the end of 7 hr. incubation at 37° the NH_2-N content of the ram semen decreased only from 3·9 to 2·9 mg. N/100 ml. In the same semen, however, there was a large evolution of free NH_3, the content of which increased from 1·3 mg. N/100 ml. in the fresh semen to 9·7 mg. N/100 ml. after 7 hr. incubation. This remarkable formation of NH_3 in semen will be discussed separately. However, it should be pointed out at once that the NH_3 formed in the semen far exceeds the amount which could be derived from the adenosine derivatives. Furthermore, the NH_3 formation is not dependent on the presence of spermatozoa but occurs also in the seminal plasma which, as was shown above, has a very low content of adenosine derivatives. Moreover, the evolution of NH_3 in the semen is not influenced by such substances as fluoride or iodoacetate or by the presence of glucose. The stability of the adenyl-amino-N content indicated that the semen must be poor in enzymes capable of deaminating adenosine derivatives. That this is so was shown by experiments designed to test the ability of whole semen, washed sperm and seminal plasma to deaminate added adenosine derivatives. There was no appreciable NH_3 production when the following sub-

stances were used as substrates: ATP, adenosine, muscle adenylic acid (adenine ribose-5-phosphoric acid) and yeast adenine nucleotide (adenine ribose-3-phosphoric acid) (Table 3). It is of some interest

Table 3. *Deaminases in semen*

(*m*-Adenylate = Na salt of muscle adenylic acid or adenine ribose-5-phosphoric acid; *y*-adenine nucleotide = Na salt of adenine ribose-3-phosphoric acid.)

Source of enzyme	Substrate 100 μg. NH_2-N	pH	NH_3-N liberated after 1 hr. at 37° (μg.)
Ram, 0·1 ml. whole semen	Adenosine	7	2
	m-Adenylate	7	4
	y-Adenine nucleotide	7	4
	ATP	7	3
	Glucosamine	7	4
	Cadaverine	7	20
Ram, 0·35 ml. seminal plasma	Adenosine	6	10
	,,	7·5	4
	m-Adenylate	6	9
	,,	7·5	12
Ram, 0·35 ml. washed sperm	Adenosine	6	0
	,,	7·5	7
	m-Adenylate	6	0
	,,	7·5	7
Boar, 0·5 ml. seminal plasma	Adenosine	7	3
	,,	7·5	5
	m-Adenylate	7	3
	,,	7·5	3
Bull, 0·5 ml. whole semen	Adenosine	7·5	5
	m-Adenylate	6	4
	,,	7·5	5

to compare this poor deaminase activity of spermatozoa and of the seminal plasma with the powerful phosphatase activity exhibited by the semen towards adenyl compounds.

Seminal phosphatases

Hitherto, work concerning the phosphatases of semen has been carried out mainly with human semen or, strictly speaking, with the human seminal plasma. Kutscher & Wolbergs (1935) discovered the high phosphatase activity of the human semen and were the first to point out that the seminal phosphatase originates chiefly in the prostate gland, from whence it passes into the semen together with the other components of the prostatic secretion. The range of activity of the prostatic phosphatase was defined by the discoverers as that of an enzyme which acts optimally at a slightly acid pH, splitting equally well α- and β-phosphoglycerol but which has only a slight effect on diphosphohexose and on pyrophosphate. Reis (1937, 1938), who tested a number of other substrates, found that a dominant characteristic of the phosphatase present in the human seminal plasma is its high activity towards certain nucleotides. He found that inosinic acid, adenylic acid and yeast adenine nucleotide are dephosphorylated several hundred times more rapidly than phosphoglycerol, and arrived at the conclusion that the so-called seminal phosphatase is in fact a complex mixture of dephosphorylating enzymes originating partly in the prostate and partly in the testicles. Reis pointed out that among the seminal phosphatases there is one which is specifically concerned with the dephosphorylation of adenylic acid. This enzyme, the '5-nucleotidase' as he called it, was shown to be highly active against adenylic acid but completely inactive towards ATP and yeast adenine nucleotide.

Using bull semen, it was possible to confirm the existence of a highly active 5-nucleotidase. In the bull seminal plasma the activity found can be expressed by the ratio

$$\frac{\text{P split from adenylic acid}}{\text{P split from } \beta\text{-phosphoglycerol}} = \frac{300}{1}.$$

The potency of the seminal plasma can be judged by the fact that from 160 μg. P added as Na adenylate to 0·001 ml. seminal plasma, 140 μg. P were found in the form of inorganic phosphate after 1 hr. incu-

Table 4. *Phosphatases in bull semen*

Source of enzyme	pH	μg. P liberated as orthophosphate in 1 hr. at 37° from 160 μg. P added as: β-Phosphoglycerol	ATP	*m*-Adenylate	*y*-Adenine nucleotide
0·1 ml. whole semen	6	25	160	160	40
	7·3	23	160	160	37
0·01 ml. whole semen	6	8	30	160	8
	7·3	8	50	160	8
0·01 ml. seminal plasma	6	7	35	160	8
	7·3	7	30	160	8
0·001 ml. seminal plasma	7·3	0·5	9	140	0
Washed sperm corresponding to 0·4 ml. semen	6	7	120	120	9
	7·3	7	140	130	9
Washed sperm corresponding to 0·04 ml. semen	6	0	35	20	1
	7·3	0	35	35	1

bation at 37°. Expressed in phosphatase units, the nucleotidase activity in the bull seminal plasma is 3000–5000 as compared with 30 in brain, 2 in skeletal muscle and 0·05 in normal serum. Yeast adenine nucleotide is dephosphorylated by bull seminal plasma much more slowly than muscle adenylic acid. ATP, on the other hand, occupies an intermediary position between the two nucleotides. Table 4 shows the phosphatase activity of the bull seminal plasma towards the various substances and the comparison between the seminal plasma and the spermatozoa. The bull spermatozoa, in spite of the fact that they have been repeatedly washed with Ringer solution, still exhibit a remarkably high phosphatase activity which is particularly noticeable with ATP. The 5-nucleotidase, on the other hand, is much weaker in the spermatozoa than in the seminal plasma. Altogether it appears that the bull semen contains at least two enzymes concerned with the breakdown of ATP. One of these enzymes converts ATP into adenylic acid, the other carries the degradation one step further and produces adenosine from adenylic acid. Analogous enzymes were also found in the semen of boar and ram. The activity here, however, is not nearly as strong as in the bull semen. The boar in particular has a low phosphatase activity which may be due to the fact that a large proportion of the voluminous semen ejaculate is contributed by the seminal vesicles. With adenylic acid as substrate, 0·01–0·03 ml. ram seminal plasma was required to bring about the same effect as 0·001 ml. bull seminal plasma. The activity of ram spermatozoa towards ATP was of the same order as that of the equivalent number of bull spermatozoa.

In view of the observation made by Winberg (1941) that spermatozoa, but not the seminal fluid, contain cozymase, the possibility has been envisaged that the bull seminal plasma, which has such a high phosphatase activity towards several adenyl derivatives, may also act on cozymase. As a matter of fact, cozymase was found to be slowly dephosphorylated both by the seminal plasma and by the washed spermatozoa of bull. Of 0·2 mg. P added as cozymase to 0·1 ml. seminal plasma and 0·4 ml. washed sperm, respectively, only 0·02 mg. P appeared as inorganic phosphate after 1 hr. incubation at 37°; 0·7 whole semen was required to set free 0·08 mg. or 40 % P after 1 hr. at 37°.

DISCUSSION

As might be expected, the two chief components of the semen, the spermatozoa and the seminal plasma, differ biochemically from each other in many respects. In conformity with their function, the spermatozoa carry several important intracellular catalysts required to maintain the respiratory activity as well as the glycolytic activity of the cells. On the high metabolic activity ultimately depends the motility of the spermatozoon and, according to some investigators, the male fertility. However, it may be pointed out here that the exact correlation between motility and fertility is still uncertain and it appears that motile spermatozoa are not always fertile (Hammond, 1930).

The respiratory mechanism of the spermatozoa has been the subject of a long dispute which centred chiefly around the question of the presence of cytochrome in the spermatozoa. Keilin (1925) has shown that among all the organs of a perfused frog two are distinguished by the highest concentration of cytochrome: the heart and the testes. Iwanow (1931) has shown that the respiration of spermatozoa is cyanide-sensitive, suggesting the probable presence of the cytochrome system, and Ball & Meyerhof (1940) detected spectroscopically the bands of cytochrome in the sperm of *Arbacia punctata*. However, so far attempts to prove spectroscopically the presence of cytochrome in mammalian spermatozoa have failed repeatedly, and the only facts from which one could deduce the functioning of the cytochrome system were experiments showing the succinic dehydrogenase and cytochrome oxidase activity of spermatozoa (Lardy & Phillips, 1941; Zittle & Zitin, 1942; MacLeod, 1943*b*). The successful demonstration in the present study of the existence of cytochromes *a*, *b* and *c* in the spermatozoa was achieved by using fairly thick suspensions of the sperm and examining them by means of the microspectroscope, an instrument particularly suited to this kind of investigation because of its small dispersion and strong illumination. There can now be little doubt that the respiratory activity of the mammalian spermatozoa is in fact maintained by the cytochrome system, and thus there is no further need to uphold the old views whereby the respiratory mechanism of spermatozoa was said to differ markedly from that of other respiring cells. There are, however, certain aspects of the semen respiration which still remain to be clarified. One of them concerns the alleged differences between the 'immature' epididymal spermatozoa and the 'mature' ejaculated spermatozoa. Then there is the problem concerning the chemical nature of the substrate for the 'endogenous' respiration of washed spermatozoa. According to Lardy *et al.* (1945), the substrate utilized by the sperm aerobically is not carbohydrate but a phospholipid; they are of the opinion that the motility of the epididymal spermatozoa is initiated and maintained by the oxidation of the phospholipid stores, which results in an uptake of inorganic phosphate and the formation of an easily hydrolyzable ester, possibly identical with ATP. Finally, it remains to be seen in what way and to what extent the seminal plasma can contribute towards the O_2

uptake of the whole semen. It is stated that in some, but not in all, mammals the sperm-free plasma can utilize O_2 (Zeller, 1941; MacLeod, 1943*a*). In view of the absence of cytochrome from the seminal plasma this O_2 uptake must be the outcome of processes entirely different from those on which depends the O_2 uptake of spermatozoa.

The glycolytic mechanism of the spermatozoa will be discussed in the next paper (Mann, 1945*b*), where it will be shown that both ATP and cozymase act as coenzymes in several intermediary reactions in the sperm glycolysis.

Gray (1928), to whom we owe the first critical study of processes underlying the senescence and decay of spermatozoa, has pointed out that the progressive loss of activity of spermatozoa may be considered from two points of view: either as a process of spontaneous and irreversible depletion of available energy or as the result of auto-intoxication whereby the products of activity inhibit the essential reactions of life. In this connexion it is interesting to comment upon the function of ATP and its position in the sperm metabolism as it emerges from the experimental findings described above. In ejaculated spermatozoa the decrease in the content of ATP coincides with the impairment of activity. In the sperm which has been deprived by washing of all glycolyzable substrate the ATP is better preserved aerobically than in N_2. This behaviour is in agreement with the well-known phenomenon that in absence of glycolyzable substances the motility of spermatozoa can be maintained much longer in air than in N_2. No definite answer can be offered at present to the question as to why the level of ATP should depend on the presence of O_2. Analogy with the metabolism of other tissues seems to justify an assumption that aerobically, but not anaerobically, the breakdown of ATP is counterbalanced by a resynthesis at the expense of the non-glycolytic aerobic processes which form part of the 'endogenous' respiration of spermatozoa. If this is at all justified, one would expect that a resynthesis of ATP should be also possible under anaerobic conditions provided that a glycolyzable substrate is added. And, indeed, one finds that when spermatozoa are incubated anaerobically in the presence of glucose, the content of ATP is preserved much better than without it. Again, this behaviour is in conformity with the observation that in anaerobically stored spermatozoa motility is maintained more satisfactorily in the presence than in the absence of a glycolyzable substrate.

SUMMARY

1. A procedure is described for the preparation of suspensions of 'washed spermatozoa' with special reference to ram and bull semen.

2. The spermatozoa are shown to contain cytochromes *a*, *b* and *c*. It is possible to follow spectroscopically the reduction and oxidation of the cytochromes and their behaviour in the presence of respiratory inhibitors. The formation in spermatozoa of a compound between cytochrome *a* and carbon monoxide is described.

3. Iron, copper and zinc occur in a higher concentration in the spermatozoa than in the seminal plasma.

4. The distribution of acid-soluble P compounds was studied. There are about 40 mg. acid-soluble P present in the spermatozoa in 100 ml. ram semen, of which 4 mg. are contributed by the readily hydrolyzable fraction. The rest is composed largely of difficulty hydrolyzable P compounds.

5. The readily hydrolyzable P fraction has been separated quantitatively as Ba salt and identified as adenosinetriphosphate (ATP). The content of ATP in ram spermatozoa is 0·6–1·5 mg. NH_2-N or 2·6–6·6 mg. labile P/100 ml. semen; the content in bull semen is 0·4 mg. NH_2-N or 1·7 mg. labile P/100 ml.

6. While the glycolytic activity of semen is restricted to the spermatozoa, enzymes which bring about the decomposition of ATP and cozymase are also found in the seminal plasma. Two enzymes at least are concerned with the breakdown of ATP. One converts ATP into adenylic acid, the other, the '5-nucleotidase', dephosphorylates adenylic acid to adenosine. Bull seminal plasma is particularly rich in '5-nucleotidase'.

7. ATP undergoes characteristic changes during the metabolic activity of ejaculated spermatozoa. Both under aerobic and anaerobic conditions, the decrease in the ATP content of the surviving sperm coincides with the loss of motility. It is suggested that the function of ATP is connected with the motility of spermatozoa.

8. In contrast to the powerful dephosphorylating enzymes, the semen lacks deaminases capable of deaminating adenyl derivatives. The considerable ammonia formation which takes place in the semen on storage depends on the presence of an enzyme system distinct from adenyl deaminases. The production of NH_3 also takes place in the sperm-free seminal plasma.

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Studies on the Metabolism of Semen

2. GLYCOLYSIS IN SPERMATOZOA

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The presence of oxygen is not an essential condition for the survival of spermatozoa. Walton, Hammond & Asdell (1928) were the first to point out that rabbit semen can be successfully stored if placed in narrow test-tubes under a thick layer of paraffin oil which protects the semen from the access of air. Shortly afterwards, Iwanow (1931) showed that dog spermatozoa suspended in an isotonic solution of glucose and phosphate retain their motility when their respiratory activity has been abolished either by poisoning with cyanide or by replacing the air with hydrogen. Redenz (1933) found that the presence of glucose or some other glycolyzable substrate is indispensable if the spermatozoa are to remain alive under anaerobic conditions; without carbohydrate the anaerobic life of the spermatozoa is very short, and in this respect the anaerobiosis differs markedly from the aerobiosis. More recently, it has been conclusively demonstrated that the metabolism of spermatozoa is predominantly of a glycolytic character, and it has been established that the glycolytic activity can serve as a useful guide in assessing the potential fertility of the semen (Shergin, 1939; Comstock, 1939; MacLeod, 1939; Lardy & Phillips, 1941; Moore & Mayer, 1941; Ross, Miller & Kurzrock, 1941; Henle & Zittle, 1942). Up to the present, studies on the glycolysis of spermatozoa were largely confined to investigations of optimal conditions, various substrates and inhibitors of the glycolysis, leaving open the question of the pathways and enzymes involved in the glycolytic processes. In this study an attempt is made to elucidate the intermediary phases in sperm glycolysis by examining the enzymes and coenzymes which control the glycolytic mechanism. It will be shown that adenosinetriphosphate (ATP) takes part in several intermediary reactions of sperm glycolysis. The evidence presented gives support to the view that ATP is the substance through which a link is established between the activity of spermatozoa on the one hand and the glycolysis on the other (Mann, 1945 *a*, *b*). However, further study is required to explore more fully the precise nature of this link.

EXPERIMENTAL

Methods

Ejaculated semen of the ram and bull was used. The spermatozoa were separated from the seminal plasma and washed with calcium-free Ringer-bicarbonate solution as previously described (Mann, 1945*b*). The suspensions of washed spermatozoa had the same density as the whole semen (ram, 2·5–5 million cells/μl.; bull, 0·7–1 million cells/μl.).

The respiration was measured in Barcroft differential manometers, in air. The results are expressed in terms of $Z_{O_2}=\mu l.$ O_2 taken up by 10^8 spermatozoa in 1 hr. at 37° (Redenz, 1933). The anaerobic glycolysis was studied by incubating the washed sperm in Thunberg tubes filled with pure N_2 or with 95% N_2 + 5% CO_2. The methods used for the estimation of lactic acid and phosphate were the same as those previously described (Mann, 1945*b*). Pyruvic acid was estimated by the method of Clift & Cook (1932). The figures for lactic acid are presented in mg. From these figures the quotient Z_L was calculated on the basis $Z_L=\mu l.$ CO_2 equivalent to lactic acid produced by 10^8 spermatozoa in 1 hr. at 37°.

Respiration and glycolysis of washed spermatozoa

Aerobically, ram spermatozoa which have been washed free of seminal plasma show a high respiratory activity. The average figure found was $Z_{O_2}=-20$ which agrees with the value reported by Lardy, Winchester & Phillips (1945). In order to convert the Z_{O_2} into Q_{O_2}, the Ringer solution was removed from the suspension of spermatozoa by washing the sperm with water and drying at 100° to constant weight. This procedure, however, is not satisfactory since it involves the use for washing of an unphysiological medium. On the whole, therefore, the use of Z_{O_2} is preferable. The average Q_{O_2} of the ram sperm based on the dry weight as determined after washing with water was −8.

The respiration of washed spermatozoa, if examined in the complete absence of seminal plasma, was not accompanied by any appreciable formation of lactic acid. On the addition of glucose, however, a glycolysis developed which was well pronounced even in a continuously aerated sperm suspension. The rate of the aerobic glycolysis of ram spermatozoa was seldom less than half of the anaerobic glycolysis, but there was much seasonal and individual variation in both the aerobic and anaerobic glycolysis. Anaerobically the highest value observed was 6 mg. lactic acid produced by 1 ml. sperm suspension ($3{\cdot}5\times10^9$ cells) in 1 hr. at 37°, which corresponds to a Z_L of +42. The lowest value observed was one-sixth of the above figure.

The initial stage of sperm glycolysis

When glucose is added to washed spermatozoa, lactic acid is formed in large amounts. Glycogen, on the other hand, yields hardly any lactic acid (Table 1). The ability of spermatozoa to utilize glucose but not glycogen resembles that of yeast where the initial step in the metabolism of glucose involves a reaction between adenosinetriphosphate (ATP) and glucose (Lutwak-Mann & Mann, 1935). This reaction is catalyzed by hexokinase and yields adenosinediphosphate (ADP) and 6-phosphoglucose (Meyerhof, 1935; Colowick & Kalckar, 1943). It can be conveniently studied in presence of fluoride which hardly affects hexokinase but helps to preserve ATP by inhibiting the adenosinetriphosphatase.

Table 1. *Formation of lactic acid from glycogen, glucose, pyruvic acid and Harden & Young ester*

(Washed spermatozoa, 10^9 cells/sample. Glycogen, 0·4%. Other substrates, 0·05 M. Total volume of samples, 1–2 ml.)

Exp. 1. Ram spermatozoa suspended in Ringer-bicarbonate buffered with 0·1 M-phosphate, pH 7·3. Incubated 1 hr., 37°, in Thunberg tubes filled with 95% N_2 and 5% CO_2.

	Lactic acid (mg.)
No substrate	0·150
Glycogen	0·165
Glucose	1·940
Pyruvate	1·435
Harden & Young ester	0·330

Exp. 2. Ram spermatozoa suspended in Ringer solution buffered with 0·1 M-phosphate, pH 7·3. To each sample added 0·2 mg. cozymase (coz.). Incubated 1 hr., 37°, in open test-tubes.

	Lactic acid (mg.)
No substrate	0·036
Glycogen	0·045
Glucose	0·289
Pyruvate	0·540
Pyruvate + glucose	1·210
Harden & Young ester	0·089
Harden & Young ester + pyruvate	1·623

Exp. 3. Conditions as in Exp. 2.

	Lactic acid (mg.)
No substrate	0·004
Glycogen	0·017
Glycogen + coz.	0·020
Pyruvate	0·690
Pyruvate + coz.	0·512
Glycogen + pyruvate	0·515
Glycogen + pyruvate + coz.	0·555

Exp. 4. Bull spermatozoa. Otherwise conditions the same as in Exp. 2.

	Lactic acid (mg.)
Glycogen	0·080
Glucose	1·580
Pyruvate	0·620
Harden & Young ester	0·335
Harden & Young ester + pyruvate	2·010

In spermatozoa, as in other animal tissues and yeast, ATP is readily dephosphorylated unless NaF is added to check the adenosinetriphosphatase activity. However, in addition to the phosphatase which acts on ATP, the semen also contains the powerful adenylphosphatase or '5-nucleotidase', so that any adenylic acid formed from ATP is rapidly broken down to adenosine. It should be pointed out, however, that the rate at which adenylic acid is dephosphorylated is smaller in the washed spermatozoa than in the whole semen; the bulk of the

5-nucleotidase is supplied by the seminal plasma and is effectively removed by washing. That the metabolism of glucose in the washed spermatozoa is in fact initiated by the shift of a labile phosphate group from ATP to the sugar is shown by the experiments recorded in Table 2. Even in absence of NaF, a slight esterification between ATP and glucose can be observed provided the time of incubation is brief and the temperature low. However, much more definite results are obtained when the reaction between ATP and glucose is examined in the presence of NaF. Thus, in Exps. 1 and 2 of Table 2, 1 ml. ram sperm, to which glucose, ATP and 0·04 N-NaF were added, brings about the disappearance of almost half of the readily hydrolyzable phosphorus of ATP within ½–1 hr. at 37°. Simultaneously, a difficultly hydrolyzable P compound is formed which behaves like 6-phosphohexose in that it can be precipitated quantitatively as Ba salt at pH 8 in the presence of 75% ethanol and which yields, after hydrolysis with N-HCl, inorganic phosphate at approximately the same rate as pure Embden ester. However, the primary products of the hexokinase reaction, namely 6-phosphohexose

Table 2. *The phosphorylation of glucose by adenosinetriphosphate*

(Washed spermatozoa. Total volume of samples 2–5 ml. Samples incubated in Thunberg tubes filled with 95% N_2 and 5% CO_2, pH 7·3, 37°. Labile P = pyro-P = that part of ATP phosphorus which appears as inorganic phosphate after 7 min. hydrolysis in N-HCl at 100°.)

Exp. 1. Ram spermatozoa. 10^9 cells/sample. Incubated with ATP (0·422 mg. labile P).

Additions	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	P_{30} (mg.)	Labile P of ATP esterified with hexose (mg.)	(%)
0	0	0·198	0·620	0·626	0	0
0·05 M-glucose	30	0·528	0·578	0·581	0·042	10
0·05 M-glucose + 0·04 N-NaF	30	0·251	0·438	0·521	0·182	43
0·05 M-glucose + 0·04 N-NaF	120	0·420	0·446	0·500	0·174	41

Exp. 2. Ram spermatozoa. 10^9 cells/sample. Incubated with ATP (0·725 mg. labile P).

Additions	Incubation (min.)	Acid-soluble P (mg.) (P_7-P_0)	Acid-soluble P (mg.) $(P_{tot.}-P_7)$
0	0	0·725	0·370
0	30	0·540	0·290
0·05 M-glucose	30	0·160	0·430
0·05 M-glucose + 0·03 N-NaF	30	0·530	0·520

Exp. 3. Ram spermatozoa. $2·5 \times 10^9$ cells/sample. Incubated with ATP (0·123 mg. NH_2-N, corresponding to 0·544 mg. labile P). In the trichloroacetic acid extract ATP + ADP were precipitated with Ba acetate at pH 8 and the precipitate analyzed for the content of NH_2-N; the supernatant solution treated with 3 vol. ethanol and the precipitate (free from pyro-P) was analyzed for content of difficultly hydrolyzable phosphate.

Additions	Incubation (min.)	Ba precipitate at pH 8: Estimated NH_2-N (mg.)	Ba precipitate at pH 8: Calculated labile P (mg.)	Ba ethanol precipitate: P-fractions determined $(P_{180}-P_0)$ (mg.)	Ba ethanol precipitate: P-fractions determined $(P_{tot.}-P_{180})$ (mg.)
0·03 M-glucose	0	0·123	0·544	0·005	0·030
0·03 M-glucose	10	0·029	0·122	0·082	0·102
0·03 M-glucose + 0·025 N-NaF	60	0·011	0·046	0·085	0·219

Exp. 4. Bull spermatozoa, $0·7 \times 10^9$ cells/sample. Incubated with ATP (0·7 mg. labile P) + 0·001 N-$MgCl_2$ + 0·013 N-NaF.

Additions	Incubation (min.)	P_0 (mg.)	P_7 (mg.)	Labile P of ATP esterified with hexose (mg.)	(%)
0·01 M-glucose	0	0·146	0·850	0	0
0·01 M-glucose	15	0·212	0·785	0·065	9
0	15	0·244	0·820	0·030	4
0·01 M-glucose	60	0·264	0·730	0·120	17
0	60	0·354	0·830	0	0

($P_{inorg.}$ = true inorganic phosphate determined as $MgNH_4PO_4$; P_0 = phosphorus determined as phosphate which reacts directly with ammonium molybdate; P_7, P_{30} and P_{180} = phosphorus which appears as orthophosphate after 7, 30 and 180 min. hydrolysis with N-HCl; $P_{tot.}$ = phosphorus as the total phosphate after incineration of the trichloroacetic acid extract.)

and ADP, do not accumulate in the spermatozoa but are further metabolized. Thus in Exp. 1, together with a sharp rise in the difficultly hydrolyzable fraction ($P_{tot.}$–P_{30}), there is also an increase in the more readily hydrolyzable fraction (P_{30}–P_7); this, as we shall see later, is due to the formation of diphosphohexose from monophosphohexose. Similarly, ADP is not stable in the sperm but undergoes further dephosphorylation as shown by Exp. 3 of Table 2. Here, the sperm was incubated for 1 hr. at 37°, with ATP, glucose and fluoride. After deproteinization, the sample was precipitated with Ba acetate at pH 8, and the Ba precipitate was analyzed for its content of NH_2-N by enzymic deamination; it was found that the NH_2-N content which represents the sum of ATP and ADP has decreased as a result of the incubation by about 90 %.

Formation of diphosphohexose

In the normal course of tissue glycolysis, 6-monophosphohexose is phosphorylated by ATP and forms the Harden & Young ester or 1:6 diphosphofructose (Ostern, Guthke & Terszakoweć, 1936).

To study the action of the phosphopherase which effects the transfer of labile phosphate groups from ATP to monophosphohexose, use was made of the Embden ester. This ester represents an equilibrated mixture of two compounds, 6-phosphoglucose and 6-phosphofructose. When added to the sperm alone, the Embden ester was hardly metabolized at all; out of 2 mg. difficultly hydrolyzable P added as Embden ester to 1 ml. ram sperm, 0·03 mg. P appeared as inorganic phosphate after 30 min. incubation at 37°, the rest remained unchanged. However, if the same quantity of Embden ester was added to the sperm simultaneously with ATP (1 mg. pyro-P) and the sample incubated 30 min., then 26 % of the Embden ester was converted into a compound which, judging by its behaviour on acid hydrolysis, was diphosphohexose or the Harden & Young ester. The amounts of lactic acid formed after 30 min. incubation, using 1 ml. ram sperm, were as follows: with Embden ester alone 0·037 mg., with ATP alone 0·057 mg., but with Embden ester + ATP 0·558 mg. lactic acid.

In view of the successful demonstration of the phosphopherase activity in spermatozoa, a study was made of the behaviour of the Cori ester or 1-phosphoglucose in spermatozoa. However, when added to the sperm, the Cori ester was hardly metabolized at all; very little or no lactic acid was produced; it was not converted to any appreciable extent either to glucose + inorganic phosphate or to Embden ester.

The conversion of diphosphohexose to phosphotriose

When 2:6-diphosphofructose is added to washed spermatozoa, it is converted to phosphotriose (Table 3) and finally to lactic acid (Table 1). A slight formation of phosphotriose from diphosphofructose is also noticeable in the seminal plasma, this in spite of the fact that the seminal plasma itself is devoid of glycolytic activity and is unable to produce lactic acid from either glucose, fructose or diphosphofructose. It appears that zymohexase, the phosphotriose-forming enzyme, although mainly concentrated in the spermatozoa, is not altogether absent from the seminal plasma. In this respect, the semen somewhat resembles the blood, where the essential glycolytic activity is restricted to the corpuscles, but the blood plasma also contains some intermediary enzymes, among them zymohexase. The rate of formation of phosphotriose in the spermatozoa and in the seminal plasma is represented in Table 3; phosphotriose has been assessed as the alkali-labile P, i.e. as inorganic phosphate which appears after exposure to 0·1 N-NaOH for 20 min. at room temperature.

Table 3. *Zymohexase activity of semen*

Exp. 1. Bull semen, diluted with 1 vol. Ringer solution, centrifuged, the dilute seminal plasma separated from the sperm, which, after one more washing, was brought with Ringer solution to the original volume of the whole semen. 1 ml. dilute seminal plasma corresponds to 0·5 ml. semen. 0·5 ml. 'washed sperm' corresponds to 0·5 ml. semen, and represents $0·3 \times 10^9$ cells. All samples contained 0·2 ml. neutral solution of Harden-Young ester (0·3 mg. P hydrolyzable in 30 min., in N-HCl). They were incubated at 37°.

	Alkali-labile P formed in presence of	
Incubation (min.)	0·5 ml. washed sperm (mg. P)	1 ml. dilute seminal plasma (mg. P)
0	0·0	0·0
5	0·02	0·0
15	0·10	0·04
60	0·15	0·16

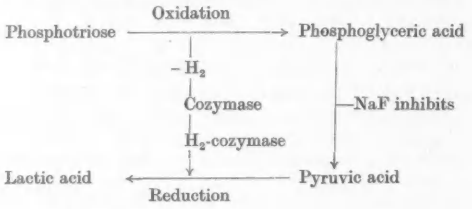
Exp. 2. Washed spermatozoa, $0·8 \times 10^9$ cells/ml., incubated at 30° with Harden-Young ester (0·3 mg. P hydrolyzable in 30 min., in N-HCl).

Sperm (ml.)	Incubation (min.)	Alkali-labile P (mg.)
0·2	0	0·0
0·2	15	0·08
0·5	0	0·0
0·5	15	0·16

The oxido-reduction between phosphotriose and pyruvic acid

The rate of lactic acid formation from diphosphohexose in spermatozoa is considerably increased by the addition of pyruvate. The mechanism underlying this process appears to be the same as in the muscle and may be defined as an oxido-reduction between

phosphotriose (derived from the enzymic cleavage of diphosphohexose) and pyruvic acid. It involves the participation of cozymase. The progress of the



reaction was studied in the following experiments. When diphosphohexose and pyruvate are added to spermatozoa and the mixture incubated in the presence of 0·04 N-NaF, the amount of lactic acid formed is as high as in the absence of fluoride. In the NaF-treated sperm, however, in addition to lactic acid there is also an accumulation of phosphoglyceric acid as indicated by the appearance of a difficultly hydrolyzable P fraction; this must be attributed to the inhibitory action of fluoride on enolase. Iodoacetate, 0·002–0·001 N., which inhibits the oxido-reduction between phosphotriose and pyruvate in yeast as well as in muscle, has a similar effect in the sperm.

The addition of cozymase to the system composed of washed spermatozoa, diphosphohexose, pyruvate and NaF, has an accelerating effect on the rate of the reaction, but even without added cozymase the reaction proceeds rapidly suggesting that the content of cozymase in the washed spermatozoa is sufficiently high to catalyze the oxido-reduction. When whole semen was used, even in the absence of NaF, the dismutation was found to proceed quite satisfactorily without additional cozymase; this may be due to an optimal concentration of cozymase in the unwashed spermatozoa; in any case, added cozymase undergoes inactivation by the seminal plasma.

In muscle and yeast, the oxido-reduction between phosphotriose and pyruvic acid is coupled with an esterification of inorganic phosphate and the synthesis of ATP (Needham & Pillai, 1937; Meyerhof, 1937; Warburg & Christian, 1939). This reaction postulates that not more than 1 mol. of inorganic phosphate should be esterified with adenylic acid for each molecule of lactic acid formed. It can be seen from the data in Table 4 that in the sperm the molecular ratio $\frac{\text{Inorganic phosphate esterified}}{\text{Lactic acid formed}}$ does not exceed 1. These experiments provide an illustration of the continuous resynthesis of adenosine-

Table 4. *The oxido-reduction between diphosphohexose and pyruvic acid and the coupling of this reaction with esterification of phosphate*

Exps. 1 and 2. Ram; washed spermatozoa suspended in Ringer-bicarbonate, 10^9 cells/ml., incubated at 37° with an equal volume of medium of the following composition, per ml.: 0·2 ml. of 0·3 M-Na-pyruvate + 0·2 ml. of 0·15 M-Harden-Young ester + 0·05 ml. of 0·1 M-$MgCl_2$ + 0·05 ml. of 0·5 % cozymase + 0·2 ml. phosphate buffer, pH 7·4, + 0·1 ml. of 0·16 M-Na adenylate + 0·1 ml. of 1 M-NaF + 0·1 ml. of 6 % $NaHCO_3$.

			Esterified P		Lactic acid		ATP			
	Incubation (min.)	$P_{inorg.}$ (mg.)	(mg.)	(10^{-5} mol.)	(mg.)	(10^{-5} mol.)	NH_3-N (mg.)	Pyro-P (mg.)	P_{180} (mg.)	$P_{tot.}$ (mg.)
Exp. 1	0	1·120	0	0	0·096	0·11	—	—	—	—
	30	0·875	0·335	1·08	1·342	1·49	—	0·28	—	—
Exp. 2	0	5·225	0	0	0·135	—	0·012	—	8·875	9·450
	30	3·850	1·375	4·35	3·890	4·30	0·330	1·407	5·545	9·350

Exp. 3. Ram; washed spermatozoa incubated with medium of the same composition as in Exps. 1 and 2, but with the exclusion of cozymase (coz.).

Additions	Incubation (min.)	P_e (mg.)	Lactic acid (mg.)	ATP-amino-N (mg.)	Adenylate converted to ATP (%)
0	0	3·55	0·18	0·003	0
0	30	3·45	1·60	0·060	11·7
0·2 ml. 1 % coz.	30	3·26	2·25	0·192	37·6
0·2 ml. coz. + 0·001 N-iodoacetate	30	4·00	0·65	0·018	3·5

Exp. 4. Ram; 0·5 ml. whole semen incubated in N_2, at 37°, diphosphohexose = diphhex., pyruvate = pyruv.

	Incubation (min.)	Lactic acid (mg.)	P_e (mg.)
Diphhex. + pyruv.	0	0·66	0·375
Diphhex. + pyruv.	60	2·13	0·505
Diphhex. + pyruv. + 0·1 ml. 1 % coz.	60	2·28	0·555
No additions	60	1·32	0·120
0·1 ml. 1 % coz.	60	1·26	0·175

triphosphate in the course of sperm glycolysis. The 'sparing effect' of the glycolysis on the content of ATP can be also demonstrated in the following way. If ATP is added to the sperm alone it disappears much more rapidly than if added together with diphosphohexose and pyruvate (Table 5). If, however, ATP and the two substrates are added together in the presence of iodoacetate, then ATP again disappears very quickly.

Table 5. *The maintenance of the ATP-level in glycolyzing spermatozoa, non-glycolyzing spermatozoa and in seminal plasma*

(Ram semen centrifuged. Seminal plasma decanted. Spermatozoa washed with Ringer solution. Both seminal plasma and spermatozoa diluted with Ringer solution up to the original volume of semen, and 1 ml. used. Pyruv. = pyruvate 0·03 M, diphhex. = diphosphohexose 0·01 M, coz. = 0·1 ml. 1 % cozymase, ATP = 0·216 mg. ATP-amino-N corresponds to 0·96 mg. pyro-P and 1·44 mg. $P_{tot.}$. Incubation at 37°, in N_2.)

Sample	Incubation (min.)	Lactic acid (mg.)	ATP-amino-N (mg.)	ATP-amino-N (% decomposition)	Inorganic phosphate set free from ATP (mg. P)
		Spermatozoa			
ATP	0	0·00	0·216	0	—
ATP	60	0·06	0·079	64	0·92
ATP + pyruv. + diphhex. + coz.	60	2·75	0·189	15	—
		Seminal plasma			
(*a*) ATP	0	1·50	0·216	0	—
(*b*) ATP	60	1·39	0·022	90	1·30
(*c*) ATP + pyruv. + diphhex. + coz.	60	1·59	0·026	88	—

Table 6. *The metabolism of phosphoglyceric acid*

(Ram; washed sperm, 3×10^9 cells/ml.; Pglyc. = 3 mg. P as Na phosphoglycerate; MA = Na adenylate (from muscle; 0·62 mg. NH_2-N and 1·37 mg. P); to all samples 0·1 ml. of 0·1 N-$MgCl_2$ added. Incubation at 37°, in Thunberg tubes filled with 95 % N_2 + 5 % CO_2.)

Exp. 1: in each sample 1 ml. sperm

Additions	Incubation (min.)	P_0 (mg.)	Pyruvic acid (mg.)	Lactic acid (mg.)	ATP-amino-N (mg.)	Adenylate converted to ATP (%)
(*a*) MA	0	0·1	0·0	0·0	—	—
(*b*) MA	30	0·2	0·02	0·27	0·007	1
(*c*) Pglyc.	0	0·2	0·01	0·04	—	—
(*d*) Pglyc.	30	0·34	0·13	0·30	—	—
(*e*) Pglyc. + MA	30	1·35	3·45	1·62	0·166	30

Exp. 2

	Sperm (ml.)	Additions	ATP-amino-N (mg.)	Adenylate converted to ATP (%)
(*a*)	1	Pglyc. + MA	0·166	30
(*b*)	0·1	Pglyc. + MA	—	6
(*c*)	1	Pglyc. + adenosine	—	3

The transfer of phosphate from phosphoglyceric acid to adenylic acid

The accumulation in sperm of phosphoglyceric acid during the oxido-reductive breakdown of diphosphohexose, in the presence of NaF, indicates that phosphoglyceric acid is an intermediary product of the sperm glycolysis.

Phosphoglyceric acid alone added to the sperm is only slowly dephosphorylated and the formation of small amounts of pyruvic acid and lactic acid can be observed. However, when extra adenylic acid is added, then phosphoglyceric acid is rapidly converted to pyruvic, and subsequently to lactic acid. The phosphate group of phosphoglyceric acid is taken up by adenylic acid and ATP is formed. When 1 ml. washed sperm of ram was incubated anaerobically for 30 min. at 37° with Na phosphoglycerate (3 mg. P), the result was 0·13 mg. pyruvic acid, 0·3 mg. lactic acid and 0·34 mg. $P_{inorg.}$. However, in the presence of adenylate the same amount of phosphoglycerate yielded 3·45 mg. pyruvic acid, 1·62 mg. lactic acid, 1·3 mg. $P_{inorg.}$, and 30 % of the added adenylate was converted into ATP. The experimental details are contained in Table 6. When adenosine was substituted for adenylic acid, only 3 %, instead of 30 %, was converted to ATP. It should be pointed out that the successful demonstration of the reaction between phosphoglyceric

and adenylic acid depends not only on the substrate concentration but also on the amount of sperm. It can be seen from Table 6 that if the sperm is reduced from 1 to 0·1 ml. ($0·3 \times 10^9$ cells), the synthesis of ATP from adenylic acid falls considerably.

DISCUSSION

The question as to what constitutes the substrate for glycolysis in spermatozoa has been studied by several investigators; considerable differences have been observed especially between different species of animals. These discrepancies may be due to the fact that the metabolism of the whole semen is not merely the outcome of enzymic reactions which take place in the spermatozoa themselves, but to a large extent it represents also the processes which occur in the seminal plasma. In ram semen, for instance, of which only half is taken up by the seminal plasma, one cannot expect to find the same conditions as in human semen, which contains a much higher proportion of plasma. For similar reasons it is not surprising that washed spermatozoa behave differently from the whole semen. For example, washed spermatozoa are hardly able to produce lactic acid from added glycogen, and yet in the whole semen of certain species, notably in human semen, lactic acid is said to be formed from extracellular glycogen. Human seminal plasma, however, is known to be particularly rich in diastase (Goldblatt, 1935) and unless the spermatozoa are thoroughly washed and the seminal plasma completely removed, there exists the possibility that the observed formation of lactic acid from glycogen is the result of an amylolytic decomposition of glycogen to glucose and a subsequent fermentation of glucose to lactic acid. It appears highly probable that free glucose forms the substrate for the lactic acid formation under natural conditions, especially as it is a normal constituent of the semen (Goldblatt, 1935). It is interesting to note in this connexion that seminal plasma is several times richer in glucose than blood serum; the concentrations of glucose (mg./100 ml.) in semen are: man 200–300 (Goldblatt, 1935), ram 217–744 (Moore & Mayer, 1941), bull 281–495 (Shergin, 1939).

In the intermediary reactions of sperm glycolysis, several points deserve comment. The experiments above show that the glycolytic pathways and enzymes involved in the carbohydrate metabolism of spermatozoa resemble those of other animal tissues and yeast. The metabolism of glucose in the sperm is initiated by the shift of labile phosphate groups from ATP to glucose. The monophosphohexose thus formed is further phosphorylated with the intervention of ATP to diphosphohexose, which is then broken down to phosphotriose and oxidized to phosphoglyceric acid. From phosphoglyceric acid, pyruvic acid is formed which is then reduced to lactic acid. The reconstitution of ATP broken up as the result of the initial reactions of glycolysis is accomplished by means of two reactions identical with those in muscle and yeast. The one occurs through the reaction between adenylic acid and the phosphate group set free during the conversion of phosphoglyceric acid to pyruvic acid; the other occurs between adenylic acid and inorganic phosphate and forms part of the phosphorylative oxido-reduction. This involves the participation of cozymase which is first reduced in the course of the oxidation of phosphotriose to phosphoglyceric acid and then re-oxidized in the course of the reduction of pyruvic acid to lactic acid.

It was previously stated that, both under aerobic and anaerobic conditions, the decrease in the ATP content of surviving sperm coincides with the loss of motility (Mann, 1945 *a*, *b*). In this study it is shown how ATP is continually broken down and resynthesized in the course of sperm glycolysis. The content of ATP, and ultimately the anaerobic survival of the spermatozoa, both depend on the maintenance of the sperm glycolysis.

Apart from establishing the existence in spermatozoa of enzymes and coenzymes concerned with the phosphorylative breakdown of carbohydrate, the experiments described above show that the mode of action of the glycolyzing enzymes can be successfully explored in the intact cells without subjecting them to such treatments as acetone extraction, autolysis, etc. They provide welcome evidence in support of the concept that the carbohydrate metabolism in the living cells runs a course analogous to that demonstrable by means of cell-free enzyme systems.

SUMMARY

1. The course of the reactions which form part of the glycolytic mechanism in spermatozoa has been explored in the cells themselves without separating the enzymes from the cell structure.

2. The study of the intermediary enzymic reactions involved in the carbohydrate metabolism of spermatozoa has shown that these reactions closely resemble those of other animal tissues and of the earlier stages of yeast metabolism.

3. Washed spermatozoa use glucose as the substrate of glycolysis. The fermentative breakdown of glucose is initiated by a hexokinase type of reaction with adenosinetriphosphate (ATP). The monophosphohexose thus formed is further phosphorylated by ATP to diphosphohexose. The breakdown of diphosphohexose follows the course through phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid.

4. Two coenzymes participate in sperm glycolysis, adenosine triphosphate and cozymase. ATP, which yields its labile phosphate groups in

the introductory phase of glycolysis, is later reconstituted (*a*) through the transfer of the phosphate group from phosphoglyceric acid to adenylic acid and (*b*) by way of a reaction between inorganic phosphate and adenylic acid, which forms part of the cozymase-catalyzed 'phosphorylative oxido-reduction'.

5. Evidence is provided in support of the view that ATP is the substance through which a link is established between the activity of spermatozoa on the one hand and glycolysis on the other.

The above work has been carried out on behalf of the Agricultural Research Council. I am greatly indebted to Prof. D. Keilin, F.R.S., for his valuable advice and criticism and to Dr A. Walton of the School of Agriculture for the considerable help he has given me throughout this investigation as well as for placing the ram and bull semen at my disposal. My thanks are also due to Mr L. E. A. Rowson for several samples of bull semen and to Miss C. Williams-Ellis for the boar semen.

Note added in proof: 27 *November* 1945. Since this paper was sent to press, the sugar present in seminal plasma has been purified and identified as *d-fructose* by means of optical rotation, reduction value and by the formation of crystalline methylphenyl-fructosazone. Washed spermatozoa can metabolize glucose and fructose alike. Yet in whole semen the natural substrate for the formation of lactic acid, as it is provided by the seminal plasma, is mainly fructose. (Mann (1945), *Nature, Lond.*, in the Press.)

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Euphorbain, a Protease Occurring in the Latex of the Weed *Euphorbia lathyris*

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(*Received* 13 *August* 1945)

Euphorbia lathyris, commonly known as Caper Spurge, is a biennial plant which grows to a height of about 3 ft. A survey of its distribution in Australia has shown that it is widespread in Victoria and New South Wales, but less plentiful in South Australia, and only rarely found in Queensland and Western Australia. When the stem or a branch of the living plant is broken near the tip, or when the stem is lanced, milky latex drips from the wound. Collection of the latex is best made in the spring, for then it flows freely.

The pH value of the latex samples examined ranges between 5·1 and 5·3, increasing by 0·5 pH units during dialysis. On addition of alkali either to the latex or acetone powder extracts (described later), a bluish purple colour appears at pH 6·5 reaching maximum intensity at pH 7·5. The colour changes to deep red as the pH approaches 9·0 and yellow at pH 11·5.

Rod-shaped crystals are visible in the latex under the microscope but they disappear when the latex is dialyzed through cellophan. Both the undialyzed latex and the dialysate give reactions characteristic of an aromatic hydroxy acid.

As reported by Ellis & Lennox (1942), the latex possesses a highly active proteinase for which the name 'euphorbain 1'* was suggested. Vines in 1903, however, referred to the work of Buscalioni & Fermi (1898), who demonstrated qualitatively the existence of a gelatinase in *E. lathyris* by placing portions

* 'Euphorbain 1' is used to distinguish this protease from that of *Euphorbia cerifera* reported by Castañeda, Gavarron & Balcazar (1942).

of the plant on a layer of gelatin and observing liquefaction.

The present paper describes some of the more important characteristics of the enzyme.

METHODS

Collection and treatment of latex. Latex was collected from a laboratory plot of *E. lathyris* by lancing the stems and allowing the latex to drip into a 10 ml. graduated cylinder. Daily collections of 2 ml. were made from 40 plants over a period of several weeks.

Before measuring its activity, the latex was diluted, unless otherwise stated, with 11·5 times its own volume of water. For dialysis, 1 vol. of latex was diluted with 4 vol. water, and dialyzed through cellophan until $KMnO_4$ was no longer reduced by the dialysate. This treatment, occupying several hours, hastened the flocculation of the latex particles which could then be removed easily by filtering through cotton-wool, before diluting the dialyzed latex to 12·5 times the volume of the original sample. Gas bubbled through the solution also favoured clotting.

In the field, 1 vol. latex was collected in 3 vol. of acetone, and the precipitate which formed was filtered, washed with acetone, dried *in vacuo* and ground finely to yield an 'acetone powder'. '0·4 % acetone powder extract' refers to an extract prepared by incubating 0·4 parts by weight of the acetone powder with 100 parts by weight of water for 1 hr. at 40°, and filtering.

Gelatinase activity (10 *min.*). This term is defined as the ratio of the reduction in viscosity which occurs during 10 min. incubation at 40° ± 0·2° of a 6·67 % (w/v) solution of gelatin containing the enzyme, to the initial viscosity. The digestion flask viscometer of Koch, Orthmann & Degenfelder (1939) was used and the procedure described by Lennox (1943) was followed.

Gelatinase activity (2 *min.*). In order to measure proteolytic activity on small volumes of liquid, a micro-viscometer was constructed which required only 3·0 ml. of the incubation mixture, containing 0·8 ml. of the enzyme solution. The enzyme and gelatin were used in the same ratio as when measuring gelatinase activity (10 min.), but a digestion period of 2 min. was adopted. Since the time of flow of the enzyme-gelatin mixture was usually between 20 and 30 sec., it was possible to make frequent measurements in the one viscometer.

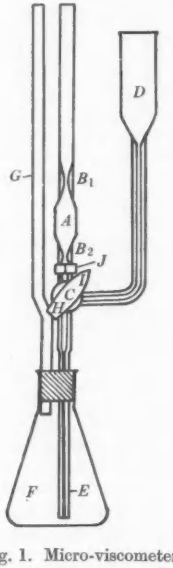

Fig. 1. Micro-viscometer used to measure gelatinase activity of small volumes of diluted latex.

As shown in Fig. 1, the apparatus comprises a viscometer bulb *A* of 0·8 ml. capacity with a constriction of 1 mm. diameter both above and below the bulb (B_1 and B_2 respectively), attached through a three-way tap *C* of 2 mm. bore to a reservoir *D*, 2 cm. diameter and 5 cm. long, with its outlet slightly above the level of the viscometer bulb. The lower connexion from the tap leads to a 0·7 mm. bore capillary tube *E*, 10 cm. long, which is attached through a rubber stopper to a 100 ml. Erlenmeyer flask *F*. The tube *G*, passing through the stopper, ensures that the air inside the flask remains at atmospheric pressure. One arm *H* of the tap *C* is ground at the tip so that it passes under the brass stop *J* attached to the stem of the viscometer. When *H* is adjacent to *J*, the three tubes from the tap are interconnected. By turning the tap anti-clockwise through 90° the reservoir is connected to the bulb, and by turning it anti-clockwise through an additional 90° the long arm *I* of the tap is brought to rest against the stop and the viscometer bulb is connected to the capillary tube. The stop enables the position of the tap to be exactly reproduced.

To measure the gelatinase activity of an enzyme, the solutions required were warmed to 40° ± 0·2° in a thermostat bath, 1 vol. 10 % (w/v) gelatin solution was adjusted to the required pH value (usually 7·0) with 0·1 vol. NaOH solution of appropriate concentration, and 0·8 ml. enzyme solution was added to 2·2 ml. of the adjusted gelatin in a test-tube at 40°. After 80 sec. the mixture was transferred to the reservoir of the viscometer, the tap being open to the bulb so that the liquid filled the bulb and rose to a level slightly above the upper constriction. The level of water in the 40° thermostat bath was kept above the level of the liquid in the viscometer. At 120 sec. after mixing the enzyme and gelatin, the viscometer tap was turned to connect the bulb of the viscometer with the capillary tube beneath and the time was taken for the surface of the liquid to pass from the upper constriction B_1 to the lower constriction B_2. The tap was then turned to allow liquid from the viscometer and the reservoir to drain into the flask before making the next measurement.

A calibration curve for the viscometer relating time of flow with viscosity in millipoises was constructed from data obtained with 40 % sucrose tested at 25 and 40°, as suggested by Koch *et al.* (1939). Gelatinase activity (2 min.) was calculated as the ratio of the reduction in viscosity of the solution containing the enzyme and 6·67 % (w/v) gelatin during 2 min. incubation at 40° to the initial viscosity.

Rennase activity. This was measured by the method of Balls & Hoover (1937) and expressed as the reciprocal of the time of clotting in minutes at 40°. The time for the formation of flecks of clot on the walls of the test-tube was taken as the time of clotting, since this interval was more sharply defined than that required for detectable thickening of the bulk of the milk. When using euphorbain there was a longer interval between these two stages than with papain.

Although the milk-clotting method is simple, rapid and requires only a small sample, the clotting time is affected by pH changes, and some activators and inhibitors could not be used in high concentration owing to their effect on the pH value. Milk powder reconstituted with 0·5 and 1·0 M buffer solutions instead of 0·1 M buffer, to increase the buffering capacity, clotted spontaneously on storage because, no doubt, of the proximity of the pH values of the final preparations to the isoelectric point of casein (4·7). The rennase activity of euphorbain preparations was found to be less reproducible than the gelatinase activity. This was primarily because the interval required for the clotting of milk could not be measured as accurately as the time of

flow of gelatin solution through a viscometer, and moreover, the pH value at which the rennase activity is measured is several units lower than the optimum value for the gelatinase activity of euphorbain, and therefore slight variations in pH may be more important.

RESULTS

Activity of material from various sources. The gelatinase activity (10 min.) of 0·4 % acetone powder extracts and of 0·4 % ethanol powder extracts, prepared from latex collected during two seasons in various parts of Victoria and New South Wales, ranged between 0·30 and 0·48. Ethanol powder extracts were prepared as described for acetone powder extracts except that the latex was precipitated with 3 vol. ethanol instead of acetone. The gelatinase activity (10 min.) of a 0·4 % extract of a medicinal grade of papain powder was 0·40. The yields of powder varied between 9 and 12 g./100 ml. *Euphorbia* latex.

Stability during storage. Acetone powder preparations retained their original activity during 12 months' storage in the laboratory. The rennase activity of a 1 in 20 dilution of the latex, on the other hand, fell from 0·26 to 0·12 during 14 days at 7°, and the rennase activity of a 0·8 % acetone powder extract fell from 0·17 to 0·05 during 12 days at 7°. The original activity was not restored by saturation with H_2S.

Optimum pH *value.* The effect of pH value on the gelatinase activity (10 min.) was studied by adding

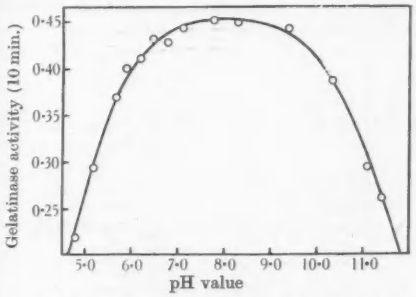

Fig. 2. pH-gelatinase activity curve for euphorbain.

the requisite amount of 0·1 M-NaOH or HCl to a portion of an acetone powder extract, and making similar adjustments to 50 ml. portions of 10 % (w/v) gelatin by reference to the titration curve for the particular grade of gelatin employed (Lennox, 1943). After mixing 25 ml. enzyme extract with 50 ml. gelatin solution and taking 50 ml. for the estimation of the gelatinase activity, the remainder was held at 40° and its pH value was measured with the glass electrode 10 min. after mixing, that is, at the same stage as the viscosity was measured for calculation of the gelatinase activity. The results in Fig. 2 show that the enzyme was most active at about pH 8, although variation in pH value between 7 and 9 had little effect.

Effect of temperature on activity. The activity of an acetone powder extract measured after storage for 17 hr. at temperatures between 7 and 55° (Table 1) shows that complete inactivation occurred at 55°.

Table 1. *Temperature inactivation of Euphorbain*

Gelatinase activity (10 min.) after 17 hr. at			
7°	22°	40°	55°
0·35	0·33	0·30	Nil

In the experiment reported in Fig. 3, the viscosity of the enzyme-gelatin mixture was measured at 4 min. intervals at each of the temperatures indicated. The 0·4 % acetone powder extract reduced

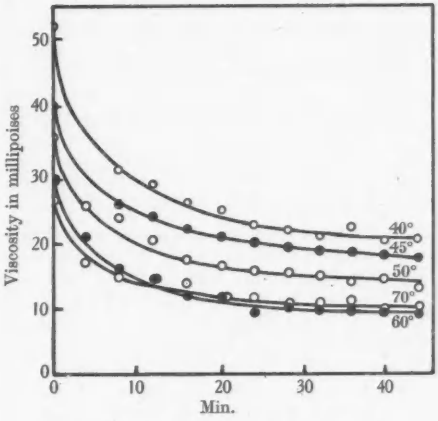

Fig. 3. Gelatin viscosity reduction curves for euphorbain at various temperatures.

the viscosity of the gelatin at all the temperatures tested, but temperature inactivation is evidenced by a flattening of the 70° curve so that, after 30 min., it failed to reach as low a viscosity as the 60° curve.

Inhibitors. As shown in Fig. 4, reducing agents did not increase the gelatinase activity of euphorbain. On the contrary, all except H_2S reduced it slightly during 20 min. incubation at 40°. Of the two oxidizing agents reported in Fig. 4, only I_2 lowered the activity appreciably, such lowering being more pronounced after dialysis than before. The loss during dialysis of material which partially protects the enzyme against inactivation is also evident from Fig. 5.

Similar results were obtained on measuring rennase activity of the latex during incubation with the same series of compounds. I_2 again produced the greatest inactivation, reducing the rennase activity from 0·40 to 0·04 within 120 sec. at 40° in the undialyzed latex, and from 0·40 to nil in the dialyzed latex under the same conditions.

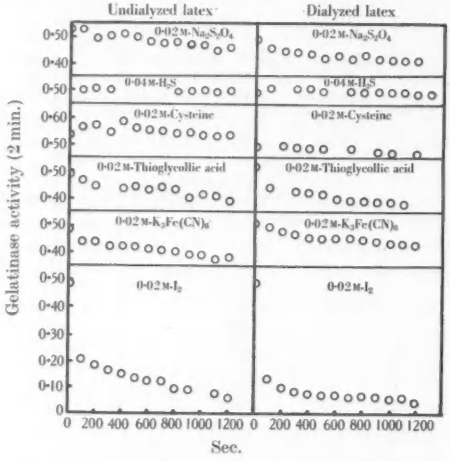

Fig. 4. Action of oxidizing and reducing agents on the gelatinase activity during incubation.

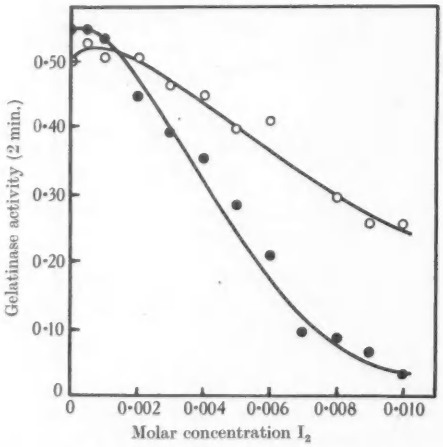

Fig. 5. Effect of I_2 concentration on gelatinase activity, o—o undialyzed latex, •—• dialyzed latex.

By increasing the concentration of I_2 in the enzyme solution and incubating for 2 min. at 40° before mixing with the gelatin, marked inactivation was again demonstrated (Fig. 5). In the dialyzed latex almost complete inactivation was obtained with 0·01 M-I_2. Attempts to restore the original gelatinase activity by half-saturating or saturating the enzyme solution with H_2S and incubating for 2 min. at 40° after inactivating with 0·01 M-I_2 for 2 min. at 40° were unsuccessful. $K_3Fe(CN)_6$ was a much less effective inactivator than I_2 for, as shown in Fig. 6, only partial inactivation was obtained

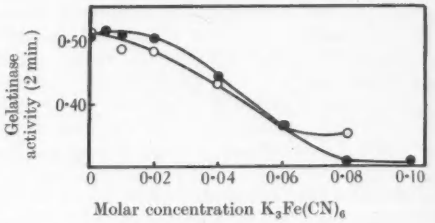

Fig. 6. Effect of $K_3Fe(CN)_6$ concentration on gelatinase activity, o—o undialyzed latex, •—• dialyzed latex.

even at 0·1 M concentration. The influence of the concentration of I_2 and $K_3Fe(CN)_6$ on the rennase activity of acetone powder extracts is shown in Fig. 7. In these experiments the inactivator-enzyme

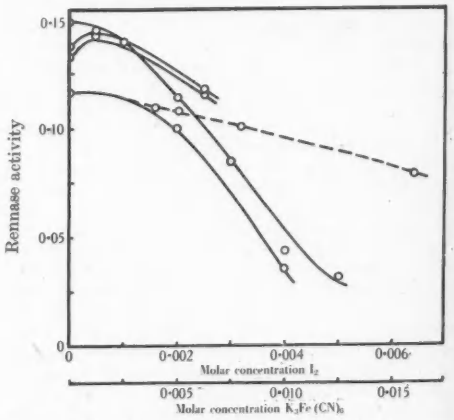

Fig. 7. Effect of I_2 concentration and $K_3Fe(CN)_6$ concentration on rennase activity of acetone powder extracts, o—o I_2 series, o- - -o $K_3Fe(CN)_6$ series.

mixtures were incubated for 30 min. at 40° before measuring the rennase activity, and the inactivation conditions were therefore more severe than those reported in Figs. 5 and 6 for the same concentrations. Slight activation of the euphorbain occurred at I_2 concentrations below 0·001 M. This is analogous to the activation of yeast endotrypsin with low concentrations of CN^- and inhibition with high concentrations, reported by Abderhalden,

Caemmerer & Pincussohn (1909). The failure to activate euphorbain with cysteine (Fig. 4) was not due to the use of too low a concentration, for dialyzing the latex in running water for 20 hr., in order to remove all traces of reducing compounds, and incubating for 2 min. at 40° with 0·002–0·08 M-cysteine did not affect the gelatinase activity (2 min.).

The inhibiting effects of ICH_2COOH, $AgNO_3$, $ZnSO_4$ and $CuSO_4$ are shown in Table 2.

Table 2. *Effect of some inhibitors at* 0·02 M *concentration on gelatinase activity* (2 *min.*)

Inhibitor	Gelatinase activity (2 min.) Undialyzed	Dialyzed
Nil	0·48	0·48
	0·48	0·47
ICH_2COOH	0·24	0·35
	0·23	0·36
$AgNO_3$	0·01	Nil
	0·02	0·01
$ZnSO_4$	0·16	0·11
	0·15	0·10
$CuSO_4$	0·03	0·05
	0·02	0·04

Evidence that the protease was not inhibited and activated by O_2 and H_2, respectively, is shown in Table 3. The Pt foil was included to catalyze any such changes, and N_2 gas was bubbled simultaneously through a third sample of latex to ensure that the control received the same agitation as the test samples.

Table 3. *Gelatinase activity after gases are passed through fivefold diluted latex for* 20 *hr. at room temperature* (15°)

(The combined latex and washings from each test were diluted to 10 times the volume of the undiluted latex before measuring the activity.)

Treatment of latex	Gelatinase activity (2 min.) O_2 passed in presence of Pt foil	H_2 passed in presence of Pt foil	N_2 passed
Undialyzed	0·54	0·50	0·52
	0·52	0·50	0·48
Dialyzed	0·47	0·48	0·49
	0·47	0·46	0·48

Action on nematodes. Attempts to digest nematodes by incubating them for 18 hr. at 40° in a 0·4 % acetone powder extract of euphorbain were unsuccessful. An extract of papain powder diluted until it had the same gelatinase activity as the euphorbain extract partially digested the same species of nematodes when tested under the same conditions.

DISCUSSION

On the evidence available the known plant proteases can be divided into two classes. The first class, comprising the papain-like proteases, possess some or all of the following properties: they are activated by reducing agents and inactivated by oxidizing agents; they act optimally, usually at pH 5, although in some instances an optimum pH value of 7 has been reported; they are more resistant to high temperatures than other proteases; and they digest nematodes and some other internal parasites. Examples are: asclepain m (*Asclepias mexicana*) (Greenberg & Winnick, 1940 *a*, *b*), bromelin (*Ananus sativus*)(Willstätter, Grassmann & Ambros, 1926 *a*, *b*), ficin (*Ficis carica*) (Walti, 1937, 1938), mexicain (*Pileus mexicanus*) (Castañeda *et al.* 1942), papain (*Carica papaya*) (Mendel & Blood, 1910–11; Bersin & Logemann, 1933; Balls, 1941; Hellerman, 1937; Maschmann, 1934), pinguinain (*Bromelia pinguin* L.) (Asenjo & de Fernandez, 1942), tabernamontanain (*Tabernamontana grandiflora*) (Jaffé, 1943 *b*), protease of *Nepenthes* (Stern & Stern, 1932), protease of yeast (Grassmann, v. Schoenebeck & Eibeler, 1931), protease of wheat (Balls & Hale, 1936), and the protease of sprouted wheat (Mounfield, 1938).

Proteases in the second class are not activated by reducing agents, but they are inactivated by heavy metals and some oxidizing agents; they act optimally at pH 8; they have a low temperature tolerance; and they do not digest nematodes. Examples are: hurain (*Hura crepitans*) (Jaffé, 1943 *a*), solanain (*Solanum elaeagnifolium*) (Greenberg & Winnick, 1940 *a*, *b*), and the protease of pumpkin (*Cucurbita pepo*) (Willstätter, Grassmann & Ambros, 1926 *a*). The properties of euphorbain show that it also belongs to the second class.

Failure to inactivate euphorbain by removal of reducing agents, by dialysis and by bubbling O_2 through the solution emphasizes its difference from ficin, which loses activity if dialyzed aerobically but not if dialyzed in an atmosphere of N_2 (Winnick, Cone & Greenberg, 1944).

Partial inactivation of the enzyme by iodine at a concentration as low as 0·002 M suggests that it is readily iodinated. Combination of enzyme with iodine has been demonstrated for pepsin (Herriott, 1936–7).

No evidence was obtained to suggest that the rennase activity of the latex was due to a separate component from that responsible for gelatinase activity. The action of inhibitors, for example, was essentially the same regardless of the method used to measure proteolysis.

SUMMARY

1. Latex collected from *Euphorbia lathyris* contains a protease for which the name 'euphorbain' has been proposed.

2. The activity is assessed by measuring the viscosity reduction effect on gelatin or by the milk-clotting method. The gelatinase activity is measured in the digestion flask viscometer or in a specially designed micro-viscometer.

3. A solid preparation which retains its proteolytic activity for at least 1 year is prepared by adding acetone to the latex.

4. Euphorbain acts optimally at pH 8, is heat-labile, is not activated by reducing agents, but is almost completely inactivated by heavy metals and by I_2 at 0·02 M concentration.

5. The activity of the latex is not affected by dialysis and it remains unaffected when O_2, H_2 or N_2 are subsequently bubbled through the solution. Dialysis, however, removes protective materials from the latex and renders the enzyme more readily inactivated.

6. Euphorbain does not fall in the papain class of proteases. Its properties resemble those of the plant proteases hurain and solanain.

The authors' thanks are due to Dr R. B. Patton of the University of Melbourne and to the various State Departments of Agriculture for information relating to the occurrence and distribution of *E. lathyris*, to Messrs M. Coleman and W. Findlay of the Victorian Department of Lands, Mr I. Armstrong and Mr B. Miller for supplying some of the latex used in this work, to Davis Gelatine Co. for supplying the gelatin, to Miss D. Doull and Mr I. S. Taylor for technical assistance, and to Mr R. A. Fookes for helping to design the micro-viscometer.

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Phosphoric Esters of the Pancreas: Choline Glycerophosphate

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(*Received* 14 *August* 1945)

Choline glycerophosphate was found by Karrer & Salomon (1926) among the products of hydrolysis of lecithin by lipase. It was stated to be a sticky, oily substance, intensely hygroscopic. We have found a similar material in the 'lead-soluble' fraction of an extract of commercial dried pancreas powder. After hydrolysis of the ester, choline and glycerophosphate were isolated.

The isolation of choline glycerophosphate resulted from an attempt to prepare choline sphingosine phosphate from the lead acetate-soluble fraction of an extract of commercial dried beef pancreas powder, according to a modification of the method previously described for fresh swine pancreas (King & Small, 1939). As a result there was obtained a fraction, of which the analytical figures were closer

to those of choline glycerophosphate than to those of choline sphingosine phosphate. Although it was not possible to isolate the substance in a pure condition, the material resembled that of Karrer, and had a choline : P ratio = 1. During the decomposition in water of the mercuric chloride compound of the ester with hydrogen sulphide and the subsequent concentration of the aqueous solution, there was a liberation of strong acid, and hydrolysis of the ester took place with a splitting-off of free choline. The resulting organic phosphorus compound was now precipitable by lead acetate. It was isolated as the barium salt (from aqueous ethanol) and proved to be glycerophosphate; the latter had presumably been formed by hydrolysis from lead acetate-soluble choline glycerophosphate.

Repetition of the preparation (twice), with careful attention to the avoidance of hydrolysis, resulted in the final concentration of a syrupy liquid, drying to a sticky hygroscopic mass, which yielded analytical figures suggestive of the possible existence of a tertiary-ester, namely a di-choline glycerophosphate.

EXPERIMENTAL

Analytical methods

Determinations of P were made colorimetrically by the perchloric acid digestion method (King, 1932); nitrogen by the micro-Kjeldahl procedure. Choline estimations were carried out by the method of Roman (1930); free choline was determined on samples freshly dissolved in water, combined choline after hydrolysis in boiling 2 N-HCl for 14 hr. Carbon and hydrogen analyses, on the material thought to be choline glycerophosphate, were by Weiler and Strauss, Oxford; analysis of the barium glycerophosphate was by Schoeller, Berlin.

Preparation of crude lead-soluble ester from pancreas

2 kg. of commercial dried beef pancreas powder were well mixed with 5 l. distilled water and heated at boiling temperature with vigorous stirring for 30 min. The mixture was made acid to litmus by the addition of 60 % perchloric acid.* After having been thoroughly chilled in ice, the mixture was poured on a large Buchner filter and the cake of precipitated protein sucked nearly to dryness.

The acid extract of the pancreas was neutralized with KOH and the precipitated $KClO_4$ removed. Basic lead acetate and ammonia were added till no further precipitate was obtained and the insoluble lead salts were filtered off. The 4 l. of filtrate were freed of lead by treatment with H_2S. The filtrate from the PbS was freed of H_2S, adjusted to pH 7, and carefully evaporated to dryness in a large balloon-flask *in vacuo*. A waxy mass remained which was only partially soluble in absolute ethanol. On concentration of the filtered ethanolic solution and addition of excess of ice-cold ether, a heavy precipitate was obtained which settled as two distinct layers. The lower yellow layer contained most of the P; the upper white layer was almost P-free. By carefully skimming off the white layer with a spatula, and repeating the process several times—solution in ethanol, precipitation with ether, settling or centrifuging in 250 ml. pots, followed by mechanical separation—a large mass of non-P containing material was eliminated. The final ethanolic solution contained 0·5 g. of organic P.

* Perchloric acid is a useful deproteinizing agent for blood (King, 1939), and is preferable to trichloroacetic acid for the preparation of acid extracts of tissue because of the great ease of disposal of excess acid in the extract through the insolubility of its potassium salt.

Isolation of choline glycerophosphate and glycerophosphate

The crude ester was submitted to the fractionation outlined in Fig. 1.

The mercuric chloride precipitates were amorphous white solids; the residues left on evaporation of the aqueous extracts after decomposition of the mercury precipitates were sticky gums. On adding ether to the ethanolic solutions of these gums oily precipitates were obtained which settled well on standing or centrifuging. The gummy residues and oily precipitates could be dried *in vacuo* over H_2SO_4 or P_2O_5 to clear yellow scaly solids, but these were very hygroscopic and some difficulty was encountered in weighing samples for analysis.

The various steps in the fractionation, outlined in Fig. 1, were followed by estimations of N, free and combined P, and free and combined choline. These estimations were performed on the solutions and on samples of the dried residues and precipitates. Precipitations of the ester with organic solvents (ether, etc.) from its solution in absolute ethanol were nearly complete and not much combined P was lost in these steps of the preparation. But in the later stages of the fractionation, when much of the contaminating material had been eliminated, the precipitation with ether tended to be less efficient, particularly if traces of water were present. It will be seen that the mercuric chloride precipitations were followed by considerable losses of combined P, N and free choline. It was not realized until several $HgCl_2$ precipitations and H_2S decompositions had been used, thattfree HCl was being formed and hydrolysis of the ester was taking place. This hydrolysis appears to have been responsible for the loss of large amounts of the ester through liberation of choline and formation of primary phosphoric ester not precipitable by $HgCl_2$. Whether the ester lost in this way was choline glycerophosphate, or whether some of it was choline sphingosine phosphate (isolated in the former preparation from fresh pig pancreas) cannot now be said.

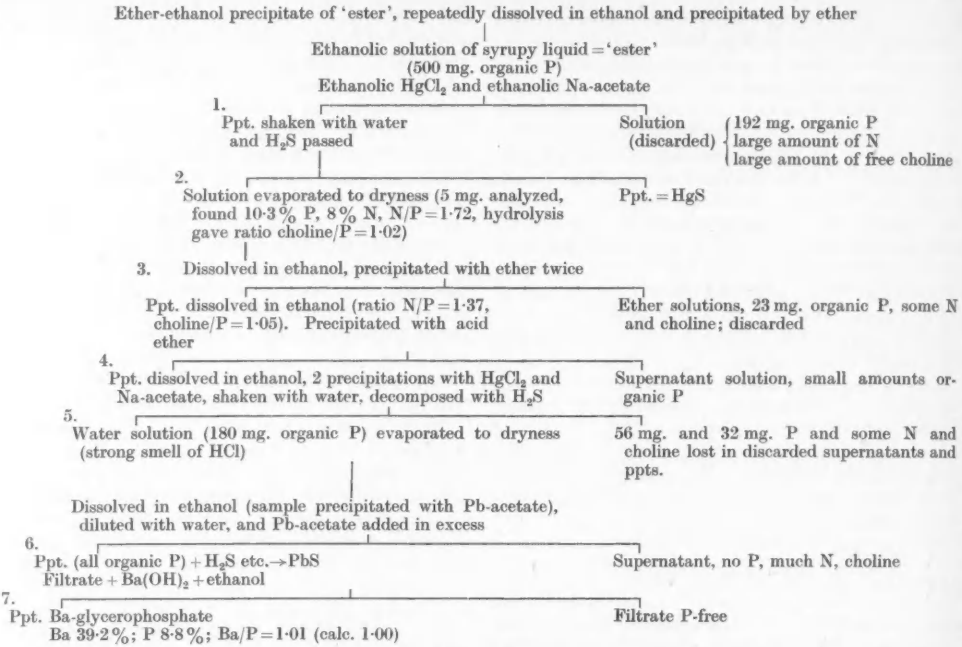
Ether-ethanol precipitate of 'ester', repeatedly dissolved in ethanol and precipitated by ether
Ethanolic solution of syrupy liquid = 'ester'
(500 mg. organic P)
Ethanolic $HgCl_2$ and ethanolic Na-acetate
1.
Ppt. shaken with water and H_2S passed
Solution (discarded)
192 mg. organic P
large amount of N
large amount of free choline
2.
Solution evaporated to dryness (5 mg. analyzed, found 10·3 % P, 8 % N, N/P = 1·72, hydrolysis gave ratio choline/P = 1·02)
Ppt. = HgS
3. Dissolved in ethanol, precipitated with ether twice
Ppt. dissolved in ethanol (ratio N/P = 1·37, choline/P = 1·05). Precipitated with acid ether
Ether solutions, 23 mg. organic P, some N and choline; discarded
4.
Ppt. dissolved in ethanol, 2 precipitations with $HgCl_2$ and Na-acetate, shaken with water, decomposed with H_2S
Supernatant solution, small amounts organic P
5.
Water solution (180 mg. organic P) evaporated to dryness (strong smell of HCl)
56 mg. and 32 mg. P and some N and choline lost in discarded supernatants and ppts.
Dissolved in ethanol (sample precipitated with Pb-acetate), diluted with water, and Pb-acetate added in excess
6.
Ppt. (all organic P) + H_2S etc.→PbS
Filtrate + $Ba(OH)_2$ + ethanol
Supernatant, no P, much N, choline
7.
Ppt. Ba-glycerophosphate
Ba 39·2 %; P 8·8 %; Ba/P = 1·01 (calc. 1·00)
Filtrate P-free

Fig. 1. Separation of lead acetate-soluble fraction from acid extract of dried pancreas.
Choline glycerophosphate, $C_8H_{22}O_7NP$ = 11·3 % P; 5·1 % N; 44·0 % choline; ratios: N/P = 1·0; choline/P = 1·0.
Choline sphingosine phosphate, $C_{23}H_{51}O_6N_2P$ = 6·43 % P; 5·8 % N; 25·1 % choline; ratios: N/P = 2·0; choline/P = 1·0.

At an early stage in the fractionation it was realized that the analytical figures being obtained, i.e. the percentages P, N and choline, and their ratios were nearer to those required by theory for choline glycerophosphate than for choline sphingosine phosphate, and the results obtained for the analyses suggested that we were almost certainly dealing with the former substance. But it was not certain that choline glycerophosphate had been present from the beginning of the process. The large amount of free choline formed and lost at step 1 could have been derived from some other ester, or from a precursor of choline glycerophosphate which contained a greater proportion of choline. (Figures suggestive of the latter possibility are presented below.)

The material thought to be choline glycerophosphate (ppt. stages 2 and 3, Fig. 1), which suffered complete hydrolysis to glycerophosphate at steps 4 and 5, was analyzed. Found: C, 35·2; H, 7·3; N, 6·8; P, 11·0 %. Calc. for choline glycerophosphate, $C_8H_{22}O_7NP$: C, 34·8; H, 7·99; N, 5·09; P, 11·3 %. The many N and P determinations performed on dried samples of the several precipitates gave figures of which the above are fairly representative; the % N was always higher, and the % P lower, than was demanded by theory, i.e. the N/P ratio was too high.

Choline was isolated from a sample of the above material, after it had been hydrolyzed with HCl, by precipitation as the platinic chloride salt, and as the periodide according to the procedure described by Booth (1935). Parts of the hydrolysis product were acetylated with acetic anhydride and with acetyl chloride, and gave typical acetyl choline responses when tested with the isolated frog's heart, and with the rectus abdominis muscle.

After the processes outlined in steps 4 and 5 had been carried out, and it was apparent that free HCl was present in considerable amount, a sample of the aqueous solution of the material was tested with lead acetate, and all the P was found to be precipitated. The whole product was thereupon treated with excess of lead acetate and ammonia, and the precipitate purified by transforming it into the barium salt and by repeated solution of the latter in water followed by precipitation with 2 vol. of ethanol. The glycerophosphate thus isolated had no optical activity when viewed in 2·1 % aqueous solution in a 4 dm. tube. Its pH titration curve showed a pK of 6·45 (found for authentic specimens of α-glycerophosphate pK 6·44, β-glycerophosphate

pK 6·34). The colour reaction of Hovey & Hodgins (1937) for glycerol was positive. Found: C, 11·9; H, 3·0; P, 9·6; Ba, 42·1%. Calc. for barium glycerophosphate, $C_3H_7O_6PBa.H_2O$: C, 11·1; H, 2·8; P, 9·5; Ba, 42·2%.

Isolation of a di-choline ester

The preparation was now repeated on a new lot of dried pancreas powder. Similar processes were followed in the fractionation, but great care was given to the maintenance at or near neutrality of all solutions. Purification was accomplished by repeated precipitation with ethanolic $HgCl_2$ and Na-acetate, extraction with water, decomposition with H_2S, concentration to dryness, solution in ethanol and precipitation with ether. Very little hydrolysis of phosphoric ester occurred, as was evidenced by the very small amounts of organic P and of free choline, in the supernatants from the $HgCl_2$ precipitations and ethanol-ether separations. The substance was finally concentrated as a syrupy liquid, which dried to a scaly, light yellow, transparent solid; it was hygroscopic and became sticky even on brief exposure to the air.

For analysis, about 24 mg. of wet material were taken. This was dried to constant weight, and then dissolved in 2N-HCl and analyzed for N, P and choline (Table 1, first fractionation). There was only a trace of free choline, and hydrolysis showed that almost all the N was represented by combined choline. But it was not certain (*a*) that the sample was completely hydrolyzed, or (*b*) that no destruction of choline had taken place during the prolonged boiling with 2N-HCl. Calculated as percentages, the figures are much closer to those demanded by theory for a di-choline glycerophosphoric ester than for the monocholine ester. The ratios of N/P and of choline/P are particularly suggestive of the existence of a tertiary ester.

Table 1. *Analyses of a 'lead-soluble' phosphoric ester (di-choline glycerophosphate?) from beef pancreas*

Fractionation of semi-pure ester	Analyses			Atomic (or molar) ratios	
	N (%)	P (%)	Choline (%)	N/P	Choline/P
First	6·1	6·8	46	1·98	1·73
Second	6·1	6·9	52	1·97	1·93
Third	6·1	6·9	45	1·98	1·67
Third recalc. to a chloride-free basis	7·0	7·8	51	—	—
Calc. for di-choline glycerophosphate	7·4	8·2	64	2·00	2·00

After a further precipitation with $HgCl_2$, etc., the material was carefully dried and another sample taken for analysis and hydrolysis (second fractionation, Table 1). The conditions used for hydrolysis in this experiment were 2N-HCl at 100° for 8 hr.

One more fractionation of the material was carried out, and the largest fraction re-analyzed. Its composition remained practically unaltered (third fractionation, Table 1). When the small weight of contained chloride was subtracted from that of the sample taken and the results recalculated on a chloride-free basis, then figures approximating to the theoretical for a di-choline glycerophosphate were obtained.

DISCUSSION

This material is still obviously impure, but the constancy of its composition on reprecipitation indicates that further elimination of contaminating substances is not likely to be attained by the type of fractionation we have employed. Furthermore, the amount of ester surviving the manipulations is too small to allow more work to be done on it. It is hoped to return to the problem of characterizing the Pb-soluble phosphoric esters of the pancreas and other tissues at a later date, when circumstances permit, and when larger batches of material can be worked up and studied. At present it appears permissible to conclude that there is probably a tertiary phosphoric ester in beef pancreas, a di-choline glycerophosphate, but proof of this must await further work.

SUMMARY

1. Glycerophosphoric acid has been isolated from the lead acetate-soluble fraction of an acid extract of commercial dried beef pancreas.

2. The glycerophosphate is thought to be derived from di-choline glycerophosphate, which is easily hydrolyzed to free choline and monocholine glycerophosphate. The latter further breaks down to choline and glycerophosphoric acid.

3. Substances approximating to the composition of both di- and monocholine glycerophosphate have been concentrated. They are intensely hygroscopic and difficult to obtain in a pure state.

The work described here was carried out in 1938–9 at the British Postgraduate Medical School and in 1939–40 at the Instituto di Pathologia, Rome. It has only now become possible to assemble the results and embody them in this communication.

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The Action of Magnesium and Calcium on the Enzymic Breakdown of Certain Adenine Compounds

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The initial breakdown of adenosine and its phosphorylated derivatives is brought about by deamination or by dephosphorylation. Deaminases for these compounds are widely distributed in the tissues although in some organs (e.g. liver) they act only after dephosphorylation (Conway & Cooke, 1939). The influence of Mg^{++} on such deaminases has not been studied in great detail. Ostern & Mann (1933) claimed that Mg^{++} inhibits the deamination of adenosine triphosphate and of muscle adenylic acid by frog muscle extract whilst, using mammalian tissue extracts, Conway & Cooke (1939), working with adenosine and muscle adenylic acid, and Brady (1942), working with adenosine, found Mg^{++} to be without effect on the deamination of these compounds. The effect of Ca^{++} on these deaminases has not, to our knowledge, been investigated.

The effect of Mg^{++} and Ca^{++} on the dephosphorylation of adenosine triphosphate has been studied more closely. The liberation of the terminal phosphate group by myosin is activated by Ca^{++} (Needham, 1942; Bailey, 1942) and is inhibited by Mg^{++} (Lyubimova & Pevsner, 1941). The removal of the second phosphate group by myokinase is activated by Mg^{++} (Kalckar, 1943); the effect of Ca^{++} has not been tested.

Our interest in this subject arose from a study of adenosine triphosphate as a metabolic factor possibly responsible for some of the general reactions to tissue trauma (Green, 1943; Bielschowsky & Green, 1943), a recent development of which was the finding that Mg^{++} potentiates the shock-inducing action of adenosine triphosphate (Green & Stoner, 1944). The present work had the object of explaining the *in vivo* findings by a study of the effects of Mg^{++} and its pharmacological antagonist Ca^{++} on the breakdown of adenosine triphosphate and related compounds *in vitro*. No attempt was made to separate the enzyme systems concerned since it was desired to approximate the conditions as closely as possible to those within the body.

METHOD

The tissues used were the liver and muscle from the albino rat. In most experiments liver slices and muscle pulp (prepared by means of a Latapie mincer) were used. The tissues were suspended in Ringer's solution prepared according to the directions of Krebs & Henseleit (1932), pH 7·4. A few experiments with watery extracts of minced liver and muscle were also performed. With the extracts, buffers of 0·01 M-$NaHCO_3$ in equilibrium with a mixture of O_2 and 5% CO_2, pH 7·1, or 0·1 M-glycine-NaOH (Kolthoff, 1932), pH 9·2, were used, although in a few experiments Ringer's solution replaced the buffer solution. Mg^{++} was added as $MgSO_4.7H_2O$ or $MgCl_2.5H_2O$ and Ca^{++} as $CaCl_2$. 'Analar' reagents and glass-distilled water were used. The reactions were carried out in Warburg vessels or small conical flasks suspended in a water-bath at 37°. The adenine compound was added in the case of the Warburg vessel from the side-arm; otherwise directly. The compounds used were the Na and Mg salts of adenosine triphosphate, the Na salts of adenosine diphosphate and muscle adenylic acid and adenosine (British Drug Houses Ltd.). All the salts were prepared from barium adenosine triphosphate and were at least 98% pure as judged by their N, pentose, total P contents and P released on acid hydrolysis for 7 min. The initial concentration in the reaction mixture was approximately 0·0004 M in every case.

After the required period of incubation the reaction was stopped by the addition of 1·0 ml. of 30% trichloroacetic acid. The precipitated proteins were filtered off immediately. The amount of adenosine radicle remaining was determined by assaying a neutralized portion of the filtrate against adenosine on the guinea-pig auricle preparation of Drury, Lutwak-Mann & Solandt (1937) as modified by Stoner & Green (1944). A biological, in preference to a chemical method, was chosen for estimating these small amounts, largely because we had the facilities available and an extensive experience of its reliability. It is highly sensitive, detecting 1 μg. of adenosine, and highly specific, for the related inosine compounds have no effect upon the guinea-pig auricle. Loss of this specific biological activity occurs after deamination and Drury *et al.* (1937), in a study of the breakdown of adenosine by blood, have already shown that loss of this biological activity is accompanied by NH_3 formation. Under our test conditions it could not be assumed that any NH_3 released would be derived from the adenine compound alone, and in view of the small quantity of the compound relative to the weight of tissue used it would be unlikely to be so. However, working with higher concentrations (0·001–0·003 M) of the adenine compounds we have found that NH_3 production runs almost parallel with loss of biological activity (Fig. 1). Consequently, under our test conditions, biological inactivation would appear to represent deamination, and throughout the paper the terms have been used as synonymous. The extent of dephosphorylation was determined by estimating the inorganic phosphate in a portion of the filtrate by Brigg's method (Peters & Van Slyke, 1932). The initial concentrations of the adenosine radicle and 7 min. P were determined by the difference between controls in which the

purine solution was replaced by an equal volume of 0·9% NaCl and those in which the reaction was stopped at zero time. The amount of deamination and dephosphorylation, i.e. the decrease in the amount of adenosine radical present and the increase in the inorganic phosphate, could then be expressed as percentages of the initial concentrations.

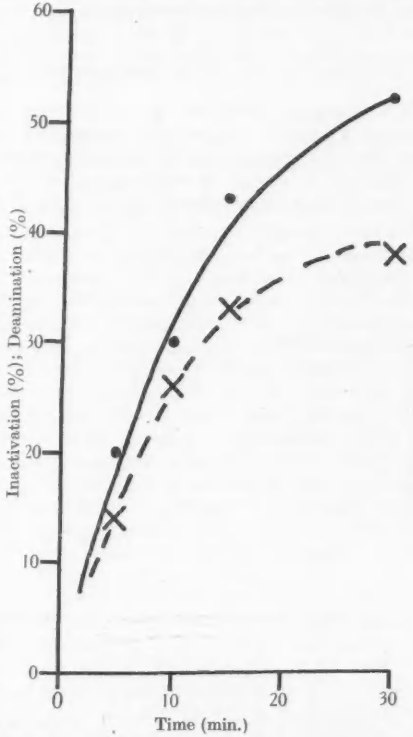

Fig. 1. Comparison of the biological inactivation and deamination, as calculated from NH_3 production, of adenosine by rat muscle. Reaction carried out in flasks under air. 300 mg. minced muscle suspended in Ringer's solution. Initial concentration of adenosine 0·0028 M. Total volume 6·0 ml. •—• % inactivation; ×----× % deamination.

RESULTS

The effect of magnesium on deamination

Under these conditions Mg^{++} proved to be a powerful inhibitor of the deaminases of adenosine triphosphate present in liver slices and minced muscle. This is shown in Fig. 2, where an increase in the Mg^{++} concentration from 3·0 mg./100 ml. (i.e. about the normal plasma level) to twice this concentration significantly delayed the deamination of adenosine triphosphate by minced muscle. A similar effect for liver extract in Ringer's solution is shown in Fig. 3, where the percentage deamination occurring in a

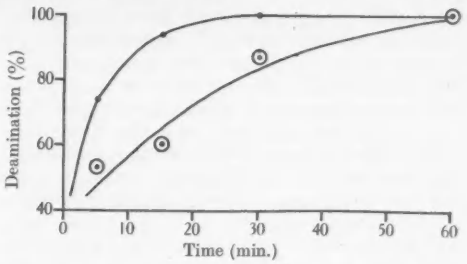

Fig. 2. Effect of Mg^{++} on the deamination of adenosine triphosphate by rat muscle. Reaction carried out in Warburg vessels under $O_2 + 5\%$ CO_2. 200 mg. of minced muscle suspended in Ringer's solution. Initial concentration of sodium adenosine triphosphate 0·0003 M. Total volume 6·0 ml.

Mg^{++} ($MgCl_2$) concentration:
•——• 3·0 mg./100 ml. (0·0013 M).
⊙——⊙ 6·0 mg./100 ml. (0·0026 M).

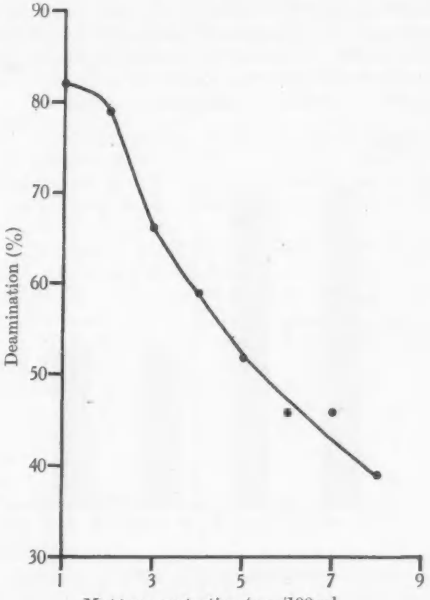

Fig. 3. Effect of different Mg^{++} ($MgCl_2$) levels on the amount of deamination of adenosine triphosphate by rat liver extract occurring in 10 min. Reactions carried out in flasks under air. Each flask contained liver extract equivalent to 200 mg. liver made up in Ringer's solution and the Mg^{++} concentration varied for each flask. Initial concentration of sodium adenosine triphosphate 0·0003 M. Total volume 6·0 ml.

standard time at different Mg^{++} levels is compared. In similar experiments with liver slices and minced muscle, curves closely resembling that of Fig. 3 were obtained. The Na and Mg salts of adenosine triphosphate were equally affected by a raised Mg^{++} level and the sulphate and chloride of Mg were equally effective as inhibitors. When no additional Mg^{++} was added to the Ringer's solution (i.e. at a Mg^{++} level of 3·0 mg./100 ml.), minced muscle deaminated magnesium adenosine triphosphate more slowly than sodium adenosine triphosphate. This may be due to a slight increase in the Mg^{++} concentration of the Ringer's solution as a result of dissociation of magnesium adenosine triphosphate. It would appear that relatively small increases in the Mg^{++} concentration above the normal plasma level can produce relatively large decreases in the rate of deamination.

Tissue extracts in the buffer solutions used were not so suitable for the demonstration of the effect. Reduction in the rate of deamination by bicarbonate (Conway & Cooke, 1939) is accentuated by the presence of Mg^{++}. Deamination diminishes with increasing pH (Conway & Cooke, 1939; Ostern & Mann, 1933). Using the glycine buffer, pH 9·2, we found little if any deamination of adenosine triphosphate by either liver or muscle extracts.

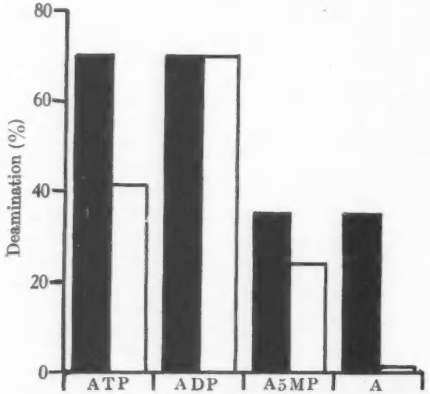

Fig. 4. Comparison of the amount of deamination of adenosine triphosphate (ATP), adenosine diphosphate (ADP), muscle adenylic acid (A5MP) and adenosine (A) occurring in 15 min. at two Mg^{++} ($MgCl_2$) levels. Reaction carried out in flasks under air containing 100 mg. minced muscle in Ringer's solution. Initial concentration in each case approx. 0·0004 M. Total volume in each case 6·0 ml. Black columns at 3·0 mg. Mg^{++}/100 ml. (0·0013 M); open columns at 6·0 mg. Mg^{++}/100 ml. (0·0026 M).

Of the other adenine compounds tested, the rate of deamination of adenosine and muscle adenylic acid by liver and muscle was inhibited by Mg^{++}, whereas under these experimental conditions, the deamination of adenosine diphosphate was unaffected (Fig. 4). The differences in the degree of Mg inhibition of adenosine triphosphate, muscle adenylic acid and adenosine cannot be stressed because of the small number of experiments.

The effect of magnesium on dephosphorylation

In this case the results were not so clear-cut. The dephosphorylation of adenosine triphosphate by liver slices and muscle pulp was definitely inhibited by $MgCl_2$ in 11 out of 14 experiments with little obvious effect in the remaining three (Fig. 5). When

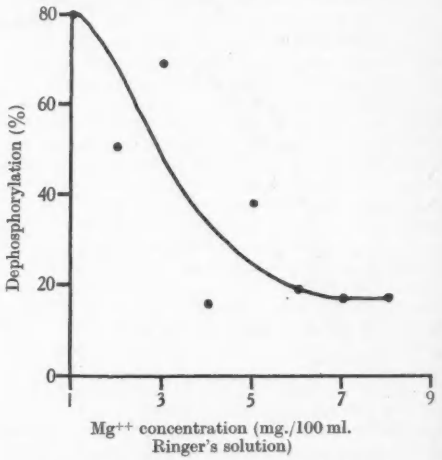

Fig. 5. Effect of different Mg^{++} ($MgCl_2$) levels on the amount of dephosphorylation of adenosine triphosphate by rat muscle occurring in 10 min. Reactions carried out in flasks under air containing 100 mg. of minced muscle suspended in Ringer's solution. Initial concentration of sodium adenosine triphosphate 0·0002 M. Total volume 6·0 ml.

$MgSO_4$ was used, the results were erratic; in seven experiments an increase of the Mg^{++} level from 3·0 to 6·0 mg./100 ml. of Ringer's solution produced slight acceleration in four, well-marked inhibition in two and no change in one. The same type of variation was seen with both liver and muscle in the form of either whole tissues or extracts and with both the Na and Mg salts of adenosine triphosphate. When no additional Mg^{++} was added to the medium (i.e. at a Mg^{++} level of 3·0 mg./100 ml.), the rate of dephosphorylation of the Na and Mg salts was the same. Compounds other than adenosine triphosphate were not studied in detail.

The effect of calcium on deamination and dephosphorylation

Briefly stated, it was found that increasing the Ca^{++} level from 12·0 to 24·0 mg./100 ml. of Ringer's solution had no effect either upon the rate of deamination or dephosphorylation of adenosine triphosphate by liver slices or muscle pulp. Compounds other than adenosine triphosphate were not studied.

DISCUSSION

Since we have shown a direct relationship between biological inactivation and deamination as judged by NH_3 production, our results would seem to indicate that under the described conditions Mg^{++} is a potent inhibitor of the liver and muscle deaminases for adenosine triphosphate, muscle adenylic acid and adenosine. The finding of Bielschowsky, Green & Stoner (1945) that larger amounts of adenosine triphosphate and adenosine given intraportally survived their passage through the liver after Mg^{++} administration suggests that the inhibition of liver deaminases produced by Mg^{++} *in vitro* may also occur *in vivo*. The cardiovascular effects of these compounds are possibly brought about during the process of deamination and we have already suggested that the diminution in these effects produced by Mg^{++} may in some degree be due to its inhibitory action on deamination (Bielschowsky *et al.* 1945). The results reported here provide some experimental support for this hypothesis.

On the other hand, our *in vitro* findings do not materially assist in explaining why Mg^{++} increases the shock-inducing effect of adenosine triphosphate and allied compounds (Green & Stoner, 1944). It was postulated (Green, 1943) that the pyrophosphate grouping in adenosine triphosphate might be responsible for its shock-inducing properties, and Bielschowsky & Green (1944) have shown that the potency of phosphorylated adenosine compounds increases with the number of phosphate groups. It seemed possible, therefore, that Mg^{++} might increase the shock-inducing effect of such compounds by delaying dephosphorylation. The *in vitro* results obtained with $MgCl_2$ on the dephosphorylation of adenosine triphosphate give some support to this view. The variable results seen with $MgSO_4$ may perhaps be explained by the work of Barrenscheen & Lang (1932), who found that the stimulant action of the $SO_4^{=}$ ion on dephosphorylation masked the slight inhibitory action of Mg^{++} ion. So far, numerous attempts to obtain consistent results in these experiments have not succeeded and until that can be done it would be unwise to interrelate the *in vitro* and *in vivo* findings more closely. The work of DuBois, Albaum & Potter (1943), in which increased yields of adenosine triphosphate were obtained from animals killed by Mg^{++} injections, suggests, however, that its action on dephosphorylation may be more pronounced *in vivo* than would seem the case *in vitro*.

Ca^{++} also increases the shock-inducing properties of adenosine triphosphate, although to a less extent than Mg^{++}, and reasons have been given (Green & Stoner, 1944) for believing that this is not a specific effect. The finding that Ca^{++} does not affect the dephosphorylation of adenosine triphosphate is not opposed to this view. Ca^{++} also potentiates the cardiovascular effects of these compounds, but again it was not thought, on physiological grounds, that enzymic processes were involved (Bielschowsky *et al.* 1945). The finding that Ca^{++} has no effect on the deamination of adenosine triphosphate is not contrary to this idea.

SUMMARY

1. The effect of Mg^{++} and Ca^{++} on the deamination (determined by a biological method) and dephosphorylation of adenosine triphosphate and other adenine compounds by liver and muscle was studied.

2. Under the described experimental conditions, Mg^{++} inhibited the deamination of adenosine triphosphate, muscle adenylic acid and adenosine, but not of adenosine diphosphate.

3. In most experiments Mg^{++}, as $MgCl_2$, inhibited the dephosphorylation of adenosine triphosphate but, as $MgSO_4$, it had a variable effect, producing chiefly acceleration.

4. Ca^{++} was without effect on either the deamination or dephosphorylation of adenosine triphosphate.

5. The bearing of these results on the action of Mg^{++} and Ca^{++} on these compounds *in vivo* is discussed.

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Substrate Specificity of Amine Oxidases

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It has been known for some time that diamines such as putrescine and cadaverine are not oxidized by amine oxidase (Blaschko, Richter & Schlossmann, 1937). This is due to their lack of affinity for the enzyme (Blaschko, 1939). Their breakdown in the mammalian organism, first studied by Udranszky & Baumann (1891), is brought about by the enzyme diamine oxidase (Zeller, 1938*a*).

The molecular grouping which is present in all substrates of amine oxidase is the amino-group attached to a terminal carbon atom. That the diamines with two such groups have no affinity to the enzyme shows that the presence of the second group in some way interferes with the ability of the first group to react with the enzyme. The nature of this interference is unknown.

We have recently had the opportunity of examining the oxidation of a number of long-chain mono- and diamines by tissue extracts and our results are described in this paper. They represent a contribution to the problem of substrate specificity.

MATERIAL AND METHODS

We wish to thank Dr H. King, F.R.S., for giving us the substances which were examined. They included two monoamines, $CH_3(CH_2)_{11}NH_2$ and $CH_3(CH_2)_{17}NH_2$ and five diamines, $NH_2(CH_2)_6NH_2$, $NH_2(CH_2)_8NH_2$, $NH_2(CH_2)_{14}NH_2$, $NH_2(CH_2)_{16}NH_2$ and $NH_2(CH_2)_{18}NH_2$. As typical substrates of amine oxidase we used tyramine, and of diamine oxidase, cadaverine. The amine oxidase preparation was from rabbit's liver (Blaschko & Duthie, 1945). As source for diamine oxidase we used pig's kidney; two preparations were employed, first, an aqueous extract of the organ dialyzed against distilled water overnight, with one-tenth the volume of M/15 sodium phosphate buffer of pH 7·4 added, and secondly, an acetone-dried preparation. The latter preparation was obtained by passing the organ through a meat mincer and grinding the mince in a cooled mortar. The brei obtained was extracted with cold acetone, three times the weight of the tissue, and this treatment was repeated three times. The last traces of acetone were pressed out and the preparation was kept in a vacuum desiccator overnight. Coarse particles were removed from the dry powder by passing it through a no. 40 mesh sieve. 1·5 g. of the resulting powder were incubated with 15 ml. of M/15 sodium phosphate buffer, pH 7·4, at room temperature for 50 min. with frequent stirring. The preparation was centrifuged for 5 min. and the supernatant, strained if necessary, in order to remove suspended particles, was used.

The manometric plan need not be discussed in detail. The standard substrate of amine oxidase was tyramine hydrochloride, used in 10^{-2} M concentration, that of diamine oxidase was $0{\cdot}5 \times 10^{-2}$ M-cadaverine dihydrochloride. The temperature in all experiments was 38°. The gas phase was oxygen in the amine oxidase experiments and air in those with diamine oxidase. The total reaction volume in each flask was 2·0 ml.

* Working with a grant from the Medical Research Council.

EXPERIMENTAL

(1) *Experiments with amine oxidase*

(*a*) *Monoamines.* The compound with 12 carbon atoms had a marked affinity for amine oxidase, and it was a substrate of the enzyme. In an experiment in which 1·6 ml. rabbit liver extract were used there was an additional oxygen uptake of 43 μl. in 15 min. with $0{\cdot}5 \times 10^{-2}$M-dodecylamine. That this was due to the amine oxidase present in the extract is supported by the observation that the amine strongly depressed the oxygen uptake due to tyramine. For instance, 0·5 ml. extract with 10^{-2}M-tyramine alone gave 90 μl. of oxygen consumed in the first 15 min., but with 10^{-2}M-tyramine plus 10^{-3}M-dodecylamine the oxygen uptake was only 4 μl. in the same period.

Octadecylamine, the other monoamine tested, gave entirely different results. There was no oxygen uptake in excess of the blank with 2×10^{-3}M-octadecylamine. This was due to a lack of affinity of the amine for amine oxidase: the oxygen uptake with 10^{-2}M-tyramine was the same in the presence and in the absence of 10^{-3}M-octadecylamine.

(*b*) *Diamines.* The substances tested fall into two distinct groups, namely, the compounds hexamethylene diamine and octamethylene diamine, and secondly, the long-chain compounds with 14, 16 and 18 CH_2 groups.

The substances of the first group had no affinity for amine oxidase; this is shown by the two experiments of Table 1.

Table 1. *Additional oxygen uptake with tyramine and diamines in rabbit liver extract*

Exp.	Addition	Extra O_2 (μl., first 15 min.)
I	10^{-2} M-Tyramine	53
	Ditto + 10^{-3} M-$NH_2(CH_2)_6NH_2$	49
II	10^{-2} M-Tyramine	49
	Ditto + 10^{-3} M-$NH_2(CH_2)_6NH_2$	49

Table 2. *Oxygen uptake of rabbit liver extract with long-chain diamines*

(1·6 ml. extract; first 15 min. readings. Substrate concentration $0{\cdot}2 \times 10^{-2}$ M.)

Extract alone	Additional oxygen uptake (μl.) with $NH_2(CH_2)_{14}NH_2$	$NH_2(CH_2)_{16}NH_2$	$NH_2(CH_2)_{18}NH_2$
7	62	42	16

The results with the three long-chain diamines were strikingly different. As Table 2 shows, all three were oxidized by the liver extracts. The initial rate of oxidation was greatest for the C_{14} compound, less for the C_{16} compound and least for the substance with 18 carbon atoms. That this oxidation was due to amine oxidase is supported by the following observations:

(1) The rabbit liver extracts contained traces only of diamine oxidase; in one experiment the extra oxygen uptakes in the first 15 min. were (μl. O_2):

With $0{\cdot}5 \times 10^{-2}$ M-cadaverine	With 10^{-2} M-tyramine	With $0{\cdot}5 \times 10^{-2}$ M-$NH_2(CH_2)_{14}NH_2$
2·5	65	47

Table 3. *Competition between tyramine and long-chain diamines*

(1·6 ml. of rabbit liver extract. Oxygen uptake for first 15 min.)

Exp.	Addition	Extra O_2 (μl.)
I	10^{-2} M-Tyramine	41
	10^{-3} M-$NH_2(CH_2)_{14}NH_2$	19
	Both the above	19
II	10^{-2} M-Tyramine	49
	10^{-3} M-$NH_2(CH_2)_{16}NH_2$	10
	Both the above	13
III	10^{-2} M-Tyramine	48
	10^{-3} M-$NH_2(CH_2)_{18}NH_2$	2
	Both the above	9

(2) The oxidation of the C_{14} diamine was not inhibited in the presence of 10^{-4} M-semicarbazide.

(3) All three substances showed competition with tyramine (Table 3). The figures given suggest that the affinity decreases in the order: $C_{14} > C_{16} > C_{18}$.

(4) Bernheim & Bernheim (1938) have separated two fractions of amine oxidase from rabbit's liver, one which is insoluble and one which is not spun down in the centrifuge. We found the ratio:

$$\frac{\text{Oxygen uptake with tyramine}}{\text{Oxygen uptake with } NH_2(CH_2)_{14}NH_2}$$

approximately the same for the two fractions. This is shown in Table 4.

Table 4. *Relative activities of rabbit liver extract and sediment in oxidizing tyramine and* $NH_2(CH_2)_{14}NH_2$

	Extra oxygen uptake (μl. O_2/20 min.)	
	Sediment	Supernatant
(A) With 10^{-2} M-tyramine	95	17
(B) With 2×10^{-2} M-$NH_2(CH_2)_{14}NH_2$	49	9·5
Ratio (A)/(B)	1·9	1·8

(2) *Experiments with diamine oxidase*

A complicating factor in the experiments with preparations from pig's kidney was the presence of both amine oxidase and diamine oxidase. This is why most of the observations were made on acetone-dried preparations, but even here a certain amount of amine oxidase activity was sometimes retained and it was therefore necessary to test the activity of the preparations towards tyramine when amine oxidase had to be ruled out as a factor in these experiments.

(*a*) *Monoamines.* The two substances with 12 and 18 carbon atoms respectively were not oxidized by the acetone-dried preparations, neither did they affect the rate of oxygen uptake with cadaverine as a substrate. Dodecylamine was tested in 2×10^{-3} M concentration, octadecylamine in $0{\cdot}5 \times 10^{-3}$ M.

(*b*) *Diamines.* As in the case of amine oxidase, two groups could be clearly distinguished, the C_6 and C_8 compounds which were found to be substrates of diamine oxidase, and the three compounds with 14, 16, and 18 carbon atoms which were not oxidized.

In one experiment with the diamines in $0{\cdot}5 \times 10^{-2}$ M concentration the oxygen uptakes (μl. O_2) during the first 10 min. were:

Extract alone	Additional oxygen uptake with Cadaverine	Hexamethylene diamine	Octamethylene diamine
5	31	20	7

This shows that in the range examined the rate of oxidation decreases with increasing chain length. Zeller (1938*b*) found that with an increasing length of the carbon chain from 2 to 5 carbon atoms there was an increase in the rate of oxidation. The rate of oxidation therefore is at a maximum in cadaverine (pentamethylene diamine). The oxidation of both hexamethylene diamine and octamethylene diamine was due to diamine oxidase because both showed marked competition with cadaverine. This is illustrated by the experiments of Table 5.

Table 5. *Competition between cadaverine, hexamethylene diamine, and octamethylene diamine*

(Acetone preparation of pig kidney. First 15 min. readings.)

Exp.	Addition	Extra O_2 (μl.)
I	$0{\cdot}5 \times 10^{-2}$M-Cadaverine	19
	$0{\cdot}5 \times 10^{-2}$M-Cadaverine $+0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_6NH_2$	10·5
	$0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_6NH_2$	12
	10^{-2}M-Tyramine	0
II	$0{\cdot}5 \times 10^{-2}$M-Cadaverine	27
	$0{\cdot}5 \times 10^{-2}$M-Cadaverine $+0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_8NH_2$	16·5
	$0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_8NH_2$	6·5
	10^{-2}M-Tyramine	0

In the aqueous extract of pig's kidney there was an oxygen uptake with the long-chain C_{14} diamine, but this was due to the presence of amine oxidase and not to diamine oxidase. With acetone-dried preparations the oxygen uptake with the C_{14} diamine was very small or completely absent, and the same was true for tyramine. The C_{16} and C_{18} diamines were also not oxidized by the acetone-dried preparations. Table 6 shows that the affinity of the

Table 6. *Competition between cadaverine and long-chain diamines*

(Acetone preparation of pig kidney. First 15 min. readings.)

Exp.	Addition	Extra O_2 (μl.)
I	$0{\cdot}5 \times 10^{-2}$M-Cadaverine	38
	$0{\cdot}5 \times 10^{-2}$M-Cadaverine $+0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_{14}NH_2$	25
	$0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_{14}NH_2$	0
II	$0{\cdot}5 \times 10^{-2}$M-Cadaverine	36
	$0{\cdot}5 \times 10^{-2}$M-Cadaverine $+0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_{16}NH_2$	34
	$0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_{16}NH_2$	0
III	$0{\cdot}5 \times 10^{-2}$M-Cadaverine	36
	$0{\cdot}5 \times 10^{-2}$M-Cadaverine $+0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_{18}NH_2$	39
	$0{\cdot}5 \times 10^{-2}$M-$NH_2(CH_2)_{18}NH_2$	0

diamines to diamine oxidase decreases with increasing chain length. In the concentration in which they were used the C_{14} acted as an inhibitor, but there was no inhibition with the C_{16} and C_{18} diamines.

DISCUSSION

Some of the results described allow us to define with greater precision the conditions of substrate specificity. The diamines with 14, 16 and 18 carbon atoms were not oxidized by diamine oxidase, but they were substrates of amine oxidase. These observations reveal one of the structural features governing the specificity of diamines for the two enzymes, viz. the length of the polymethylene chain. With short chains (up to C_8) the second amino-group interferes with the affinity for amine oxidase, but with long chains (e.g. C_{14}) the interference disappears and substrate specificity appears. Conversely, short chains (up to C_8) appear to be necessary for substrates of diamine oxidase. That the C_{14} diamine inhibits the oxidation of cadaverine may indicate that the loss of affinity for diamine oxidase occurs at a greater chain length than the loss of substrate specificity.

A full analysis would require the study of the diamines with an intermediary chain length, which were not at our disposal. But it seems likely that the results so far obtained find their explanation in the different molecular orientation required for substrates of the two enzymes. Zeller (1941, 1942) had already postulated that for oxidation by diamine oxidase it was necessary for both amino-groups of the substrate molecule to react with the enzyme and that this would require an arrangement of the long axis of the molecule parallel with a surface of the enzyme. Such an orientation of short-chain diamines is to be expected on surfaces, e.g. on an oil-water interface; and it would be a necessary condition for the diamine oxidase reaction to occur. With an increasing length of the carbon chain influences which tend to lift the carbon chain off the surface become stronger, viz. the Coulomb forces of attraction between $-CH_2-$ groups of neighbouring carbon chains. These forces, being additive, increase with increasing length of the chain, and in the diamines with 14, 16 and 18 carbon atoms an orientation vertical to the surface might explain why these substances are not substrates of diamine oxidase. In the case of amine oxidase the position would be exactly reversed. The attachment of an amine to this enzyme seems to require an orientation in which the carbon chain stands vertical to a surface. The rate of oxidation of typical substrates of amine oxidase is not affected by the presence of short-chain diamines; this shows that amines which have a tendency to orientate themselves parallel to the surface are not bound by the enzyme.

SUMMARY

1. The oxidation of two straight-chain monoamines, dodecylamine and octadecylamine, and of five diamines with 6, 8, 14, 16 and 18 CH_2 groups by

amine oxidase (rabbit liver) and by diamine oxidase (pig kidney) has been studied.

2. Dodecylamine is oxidized by amine oxidase, whereas octadecylamine is not oxidized.

3. Of the straight-chain diamines, hexamethylene diamine and octamethylene diamine are substrates of diamine oxidase; the three diamines with 14, 16 and 18 carbon atoms are not oxidized by the enzyme.

4. The diamines with 14, 16 and 18 carbon atoms are substrates of amine oxidase.

5. The specificity of substrates of the two enzymes is discussed in connexion with these results.

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The Colouring Matters of Galls. 2

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Whereas it is comparatively easy to collect galls produced by insects, the collecting of galls formed by fungi offers greater difficulties. Furthermore, these galls have not been so extensively studied as insect-galls (Guttenberg, 1905). Thanks to Dr von Wehner, the nephew and assistant of the late Dr von Guttenberg, we were able to obtain reasonable quantities of the six galls investigated by his uncle, which Dr von Wehner collected for us in the vicinity of Graz. Galls of *Ustilago maydis* Guttenberg, on maize collected by one of us at Tifton, Georgia, U.S.A., were found to contain the same pigment as the Austrian galls.

EXPERIMENTAL

The pigments were prepared, identified and investigated as described by Nierenstein & Swanton (1944). They proved known colouring matters described by Nierenstein & Swanton, whose preparations we used for mixed melting-points. The yields in all cases were poor, the maximum obtained being 0·27 %.

Analytical and descriptive data

Melampsorin. Yellow needles, m.p. and mixed m.p. 233–237° produced by *Exoascus amentorum* H. Schneider on the fruits of *Alnus incana* Web. (Found: C, 50·7; H, 5·2; purpurogallin, 40·5; glucose, 63·7 %; mol. wt. 539, 551. Calc. for $C_{23}H_{28}O_{15}$: C, 50·7; H, 5·2; purpurogallin, 40·4; glucose, 63·2 %; mol. wt. 544.)

Raestelin. Red needles, m.p. and mixed m.p. 242–244°, isolated from the galls produced by *Albugo candida* Pers. on the leaves of *Capsella bursa pastoris* L., by *Exobasidium rhododendri* Cram. on the leaves of *Rhododendron hirsutum* L. and *R. ferrugineum* L. (Found: C, 50·6, 50·7, 50·4; H, 5·3, 5·3, 5·4; purpurogallin, 40·5, 40·4, 40·5; glucose, 63·0, 63·3, 63·1 %; mol. wt. 555, 573; 541, 549; 551, 587. Calc. for $C_{23}H_{28}O_{15}$: C, 50·7; H, 5·2; purpurogallin, 40·4; glucose, 63·2 %; mol. wt. 544.)

Xestophanesin. Dark red needles, m.p. and mixed m.p. 265–266°, produced by *Puccinia adoxae* Haberl. on the leaves, twigs and buds of *Adoxa moschatellina* L. (Found: C, 50·8; H, 5·5; purpurogallin, 40·4; glucose, 63·1 %; mol. wt. 551, 562. Calc. for $C_{23}H_{28}O_{15}$: C, 50·7; H, 5·2; purpurogallin, 40·4; glucose, 63·2 %; mol. wt. 544.)

Pontanin. Yellow needles, m.p. and mixed m.p. 283–287° produced by *Ustilago maydis* Guttenberg on the leaves of *Zea mays* L. (Found: C, 50·6, 50·8; H, 5·3, 5·2; purpurogallin, 40·5, 40·4; glucose, 63·0, 63·2 %; mol. wt. 548, 554; 547, 562. Calc. for $C_{23}H_{28}O_{15}$: C, 50·7; H, 5·2; purpurogallin, 40·4; glucose, 63·2 %; mol. wt. 544.)

In conclusion we draw attention to the use of these colouring matters as low-range indicators (Nierenstein, 1945).

SUMMARY

The isolation and characterization of colouring matters from various galls produced by fungi is described.

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The Effect of pH on Stem-end Blackening of Potato

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Potatoes of certain stocks when heated for a sufficient length of time and then exposed to air develop a greyish black discoloration. This occurs mainly at the stem or basal end of the tuber and is known as stem-end blackening. The minimum time of heating is said to be 2 min. (Robison, 1941); this is sufficient to cause slight greyness only and a longer period of heating leads to a greater amount of blackening. The usual method of inducing formation of the pigment for estimation of its quantity is to cook the potato tissue in boiling water (Cowie, 1942; Rieman, Tottingham & McFarlane, 1944; Smith, Nash & Dittman, 1942); this method has been used in the present investigation.

The chemical nature of the pigment and of its precursors is unknown. It seems fairly certain that it is not melanin (Robison, 1941; Nutting, 1942); the pigment does not develop in acid solutions (Tinkler, 1931; Robison, 1941; Smith *et al.* 1942; Nutting & Pfund, 1942), but no quantitative data are available nor is the cause of this effect clear. Strong reducing agents are reported to decrease the extent of development of the colour (Smith *et al.* 1942)—it does not develop in the absence of oxygen (Tinkler, 1931; Nutting and Pfund, 1942; Robison, 1941)—and from this aspect the addition of sodium sulphite to the potato was tried but it was found ineffective in reducing discoloration (Wager, Tomkins, Brightwell, Allen & Mapson, 1945). Variety and agricultural conditions influence the liability of tubers to blacken (Wallace, 1921; Findlay, 1928; Cowie, 1941, 1942; Smith *et al.* 1942; Rieman *et al.* 1944, etc.); this has also been studied and the results will be reported elsewhere.

METHODS

Strips of dried potato were utilized in most of this work. They were prepared by cutting the raw tuber into strips $\frac{1}{4} \times \frac{1}{4}$ in. in cross-section, washing them, 'scalding' them in boiling water long enough to inactivate the enzymes and drying in a current of hot air at 60° (see Wager *et al.* 1945). The behaviour of such material with regard to colour and pH is similar to that of strips of raw potato. It can be stored for long periods with no detectable change and the use of 10–20 g. of strips per test eliminates most sampling variation, which is relatively large as between different batches of raw tubers taken from the same stock.

The short period of boiling ($1\frac{1}{2}$–2 min.) necessary to inactivate the enzymes before drying causes only a partial development of stem-end blackening pigment. To complete this development the strips were soaked for 1 hr. at room temperature in a phosphate-citrate buffer, boiled until cooked (5–10 min.), drained and left for not less than 30 min. to allow maximum development of pigment.

The greyness of the cooked samples was estimated by a group of experienced 'judges' and their individual estimates averaged. Colorimeters were not used because of difficulty in reducing different samples of potato tissue to a homogeneous mash in a comparable physical state, especially with regard to the content of air and water. Samples were awarded marks on a scale of 4 (very grey) to 0 (not grey).

The pH of a sludge of cooked strips in distilled water was found to differ little from that of the solution in which they were cooked and therefore the pH of this solution has been used as an estimate of that of the cooked tissue.

RESULTS

The effect of pH *on the colour of cooked potato.* The relationship between the greyness of cooked strips of potatoes from different stocks and varieties and their pH is shown in Fig. 1. The curves all show a similar increase in mark for greyness with increase in pH from the point at which the greyness mark attains a value of 0·4–0·5, but the pH at which this occurs differs markedly in different stocks. Below this level the marks for greyness are a less reliable estimate of stem-end blackening since other minor discolorations (such as traces of melanin, etc.) unaffected by pH in this range contribute to the greyness mark. For this reason the shape of the curves below a greyness mark of 0·4–0·5 is disregarded. At pH values greater than 7·5–8·0 the grey colour of the strips changes to a brown and this tendency increases as the pH is increased.

The greyness of strips cooked in buffer solutions is independent of their colour before cooking, e.g. potato scalded in an acid solution made pale yellow dried strips but when scalded in a slightly alkaline

Table 1. *Greyness of potato strips scalded at* pH 7·6 (*Sample A*) *and* pH 5·7 (*Sample B*) *and cooked at varying* pH*'s*

pH of solution used for cooking	Greyness	
	Sample A	Sample B
4·8	0·0	0·2
5·8	0·2	0·2
6·8	1·7	1·4
7·5	2·2	2·2

solution the dried strips were of a grey colour; both batches were, however, equally grey when cooked in buffer solutions (Table 1).

The greyness is due, therefore, to a pigment (or to a group of several pigments) whose colour is reversibly affected by pH and the quantity of the pigment (or pigments) formed in any given stock appears to be independent of pH.

If different pigments or different proportions of the same group of pigments were present in different varieties of potato, the curves in Fig. 1 would be

Table 2. *Effect of initial melanin on greyness of cooked potato strips*

pH of cooked strips	Greyness: Strips containing melanin	Greyness: Control
3·5	2·1	0·0
5·2	2·5	0·1
6·1	2·8	0·2
7·0	2·9	0·7
7·6	3·2	1·2

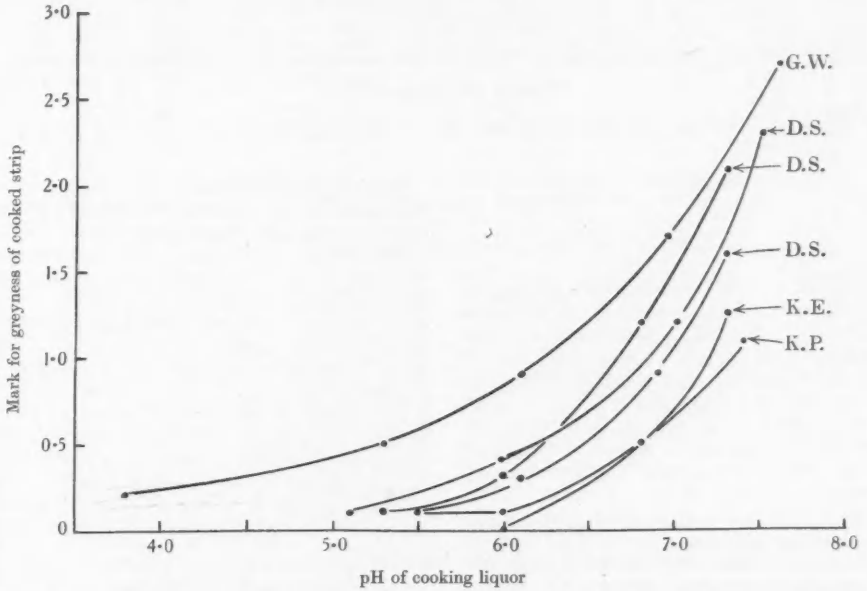

Fig. 1. The relation of the pH of the cooking liquor to the greyness of cooked dried potato strips from stocks of Golden Wonder, Doon Star, King Edward and Kerr's Pink potatoes.

likely to diverge. Since they are as nearly parallel above a greyness mark of 0·4–0·5 as could be expected from visual estimates, they offer no evidence for the existence of more than one pigment either in different stocks or varieties.

Stem-end blackening pigment and melanin. Strips of potato were allowed to discolour by enzymic oxidation before scalding so that grey-coloured dried strips were obtained. A control batch of dried strips was also made. Both batches were cooked in solutions with a range of pH. The results are shown in Table 2.

The approximately constant difference between the marks awarded to the two sets of strips is to be attributed to melanin which is unaffected by changes of pH in this range. This is direct evidence that the stem-end blackening pigment is not of the melanin type; indeed the proportion of both present may be readily estimated by alteration of the pH of the tissue.

The effect of pH *on an extract of stem-end blackening pigment.* The same degree of discoloration, due to stem-end blackening, may occur at pH values ranging from 4 to 7 in different stocks. If this discoloration is due to varying amounts of one pigment in different stocks, then this pigment must (*a*) be grey in colour when at pH values of from 4 to 7 and (*b*) increase progressively in intensity of greyness over the same range of pH. This would result in strips from a stock with a low content of pigment being graded as nearly white until they attained a high pH value and conversely.

To test this an aqueous extract of cooked dried strips from a blackening stock of potatoes was

partially purified by repeated precipitation of impurities with ethanol and concentration at 40° under reduced pressure. A solution was thus obtained which was pale brown-yellow when acid, brownish blue in the pH range of about 4–7·5 and bright yellow-brown when alkaline (pH 11–12). Portions of this were adjusted to pH values ranging from 1·5–9·5 by the addition of HCl or NaOH. The absorption of light by these solutions was estimated with a Zeiss Pulfrich photometer using a range of colour filters. The curves in Fig. 2 show the values

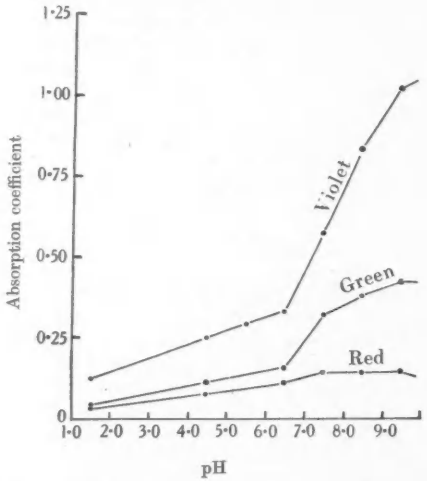

Fig. 2. The relation between the pH of a solution of stem-end blackening pigment and the absorption coefficient for three wave-bands. Wave-bands of violet filter <460 mμ maximum (approx.) 420 mμ. Wave-bands of green filter 507–555 mμ maximum (approx.) 540 mμ. Wave-bands of red filter >640 mμ maximum (approx.) 680 mμ.

of the absorption coefficient for three wave-bands (violet, yellow-green and red) as related to the pH of the solution. The graph shows that the composition of the transmitted light varied little over the pH range of 1·5–7·5, while at pH values above this the solutions became relatively redder. The intensity of coloration, shown by the magnitude of the absorption coefficient, decreased rapidly from pH 9·5 to 6·5 and thereafter more slowly.

As a further check on the relative effects of change of pH of the solution and of concentration of the pigment on the composition of the transmitted light, a solution was diluted five times at a pH of 8·5 and compared with an undiluted solution at a lower pH. The results are shown in Table 3. The discrepancies in the two sets of readings are well within the experimental error.

Table 3. *Relative effects of change of* pH *and concentration on composition of transmitted light*

	Absorption coefficient	
Filter used	Diluted solution pH 8·5	Undiluted solution pH 3·7
Violet	0·42	0·42
Blue	0·30	0·31
Blue-green	0·23	0·24
Green-blue	0·19	0·18
Yellow	0·17	0·16
Light red	0·16	0·15
Dark red	0·11	0·12

Extracts were prepared from cooked strips of a blackening stock of Golden Wonder and of Kerr's Pink potatoes. The absorption of light by solutions of these at pH values from 6·5–12·0 was determined. Over the whole of this range and for seven wave-bands of light the differences in absorption were small, such as could well have been produced by traces of other pigments (melanin, etc.). Thus, again, there is no evidence that more than one pigment is concerned in stem-end blackening.

The behaviour, therefore, of relatively pure extracts of the pigment accords with the assumption that greyness of cooked potato is due to the presence of one pigment in different amounts in different stocks. This view is further supported by the similarity in shape of the curves in Fig. 1 and of those in Fig. 2.

SUMMARY

1. The greyness of cooked potato has been estimated at a range of pH values. A rapid increase in greyness with pH above a certain value, characteristic of each stock, was observed. The absorption of light by a water extract of stem-end blackening pigment at a range of pH values has been measured.

2. It is concluded that stem-end blackening is caused by a single pigment whose colour is reversibly affected by pH and that variation in the degree of blackening between different stocks is due to the presence of different amounts of this pigment.

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Chromatography in Aqueous Solution with Mineral Precipitates Insoluble in Water

1. APPLICATION TO THE SEPARATION OF AMINO-ACIDS BY ADSORPTION ON Ag_2S

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The application of chromatography in aqueous solution has not yet been fully explored, owing to the novelty of the technique and to the empirical character of the work to which it has given rise. Experimental considerations have so far limited its use to a fairly miscellaneous choice of adsorbents, the composition and chemical properties of most of which are not well known (silicates, activated carbons, synthetic resins, etc.). The only exception is alumina. Silica gel, which also is a chemically well-defined adsorbent, in most cases functions only as a support (Martin & Synge, 1941). The time has now come to free this technique from its present empiricism and to define its sphere of application. We hope that this work will be a contribution in this direction.

At first sight, the utilization of the mineral precipitates commonly available in the laboratory, in chromatography, does not seem to be of particular interest. Alumina appears to constitute a special case; its adsorbent properties with respect to cations, and perhaps to anions, are due to the impurities which it contains. The impurities can accentuate corrosion during activation of the adsorbent by an acid and thus considerably increase the surface. The fact that the use of the mineral precipitates has so far been systematically avoided is equally significant. According to Schwab (Böttger, 1939), they are not suitable, on account of their lack of selectivity and of their too weak adsorbing power.

To assess the interest of the question, however, it suffices to compare the adsorbent properties of the usual adsorbents and of mineral precipitates. The latter, prepared in presence of an excess of one of the ions of the lattice, show a heterogeneous surface structure entirely similar to that of an 'acid' column of alumina (Fajans & von Beckerath, 1921; Kolthoff, 1934); their adsorbent properties are very well known, thanks to studies in adsorption (Fajans, 1941; Fajans & Erdey-Gruz, 1931; Kolthoff & MacNevin, 1936; Kolthoff & Rosenblum, 1933 *a*, *b*; Kolthoff, Von Fischer & Rosenblum, 1934), in analytical chemistry (Fajans & Wolff, 1924; Burnstein, 1927; Kolthoff & Lingane, 1936), in colloidal chemistry (Verwey, 1933; Verwey & Kruyt, 1934), to studies utilizing radio-active elements (Hahn, 1926, 1930; Paneth, 1914), and studies on photographic sensitivity (Sheppard, Lambert & Walker, 1939; Trillat & Aubry, 1941; Trillat & Merigoux, 1941). It must be remembered that the determining factor of the adsorption is the insolubility of the adsorption compound which the adsorbed ion forms with an ion of the opposite sign, which is part of the lattice or is itself adsorbed on it (Fajans & von Beckerath, 1921). After the precipitate is washed, the amount of excess ion of the lattice adsorbed is very slight, even in the case of substances which are sparingly soluble; the adsorption phenomena to which they give rise are only observable with solutions of radio-active elements (Kolthoff, 1934, 1936). We shall show, nevertheless, that their adsorptive power is still sufficient to allow of their utilization in chromatography in aqueous solutions.

We have attempted to separate mixtures of natural amino-acids by chromatography on a precipitate of silver sulphide.* Various chromato-

* A preliminary note on this subject has already been published (Hamoir, 1943).

graphic methods discovered in the last few years for separating natural amino-acids have already made possible the isolation of the basic acids (Turba, 1941; Wieland, 1942 *a*, *b*; Freudenberg, Walch & Molter, 1942), the dicarboxylic acids (Turba & Richter, 1942; Wieland, 1942 *a*, *b*; Wieland & Wirth, 1943), and the mono-amino monocarboxylic acids (Karrer, Keller & Szönyi, 1943; Schramm & Primosigh, 1943; Turba, Richter & Kuchar, 1943; Wieland, 1942*c*; Gordon, Martin & Synge, 1943, 1944). But certain separations are still in need of improvement; the methods are not always sufficiently reliable (separation of the dicarboxylic acids), and sometimes their accuracy leaves something to be desired. We have chosen as adsorbent a flocculate of Ag_2S, because of its very great insolubility (which results in the formation of a precipitate with a large surface and implies a high adsorption energy of the ions of the lattice), and because nearly half the natural amino-acids form insoluble silver salts which are relatively well characterized.

METHODS

Preparation of the adsorbent and of the adsorption columns. The sulphide precipitate is prepared by passing an excess of H_2S into 2 l. of a solution of silver nitrate or sulphate (equivalent to 12·5 g. as sulphide), and then leaving for several hours in the presence of the saturated solution of H_2S. The excess H_2S is then completely removed, and the precipitate carefully pulverized in a mortar. Thereafter, CO_2-free water is used. The precipitate is left for about 3 hr. in the presence of a dilute solution of 1–2% silver nitrate, then washed till the reaction with NaCl is perfectly negative. This is the activation process.

For preparing the adsorption columns, tubes with a sintered glass bottom (Allihn, Schott & Gen., Jena) are used, permeability 2, 3 or 4, into which the precipitate is packed by suction (Fig. 1). The porous plate should be very clean; after a certain time it should be washed with a concentrated solution of KCN, even if the permeability does not seem to be lessened. The columns obtained are completely homogeneous in spite of the size of the grains, and whatever the height (generally 2–3 cm., sometimes 10 or more). The method of operation is simple, rapid and easy.

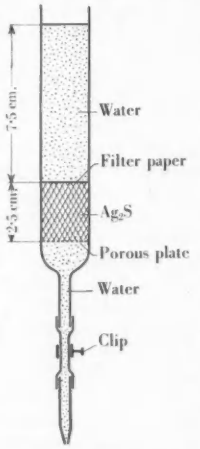


Fig. 1. Diagram of adsorption column.

Methods used for quantitative determination. The adsorption experiments have been followed by means of certain very sensitive colour reactions performed on the filtrate after concentration *in vacuo*, and by Van Slyke's micro-method for determining amino-nitrogen (Van Slyke, 1929). We have not been able to reproduce the precision of 0·5 μg. obtained by this author; we have observed slight variations in the blanks and in determinations of 1·5–3 μg. As the quantities to be determined in the course of our separation tests were only about 50 μg., the errors amount to a few per cent, but the method is sufficiently accurate to establish the quantitative character of the technique.

Attention should be drawn to certain causes of error: (*a*) the presence of moulds affects the determination adversely, their reducing power causing an additional liberation of nitrogen; (*b*) phenolic derivatives, such as thymol form, under Van Slyke's conditions, a *p*-nitroso derivative, then an unstable diazo derivative; (*c*) an aqueous solution of acetic acid concentrated under reduced pressure at 50° in the presence of traces of a heavy metal (Mn) decomposes, forming compounds which liberate traces of nitrogen or other inert gas in the Van Slyke amino-N determination.

RESULTS

Properties of the adsorbent

A flocculate of silver sulphide adsorbs irreversibly considerable amounts of silver ions which in turn adsorb those anions forming sparingly soluble silver salts. In practice, a column of 12·5 g. of sulphide does not retain histidine, but after activation, it adsorbs quantities of the order of 1 mg. Any sparingly soluble precipitate activated by adsorption of one of the ions of the lattice can, therefore, judging from the behaviour of silver sulphide, be used for chromatography in aqueous solution. The ratio of the quantity adsorbed to the weight of adsorbent for Ag_2S (1/10,000) places these substances among the adsorbents which are relatively only slightly active; for the usual adsorbents, the ratio is 1/1000. On the other hand, the adsorbing power both of different preparations, and of the same preparation at different times, is very constant; no change in the quantity of histidine fixed is observed by modifying the temperature of preparation (by working at 0°), the speed of precipitation (by accelerating the passage of H_2S or by mixing quickly a solution of sodium sulphide and silver nitrate), and the conditions of precipitation (by using or not using mechanical agitation). Further, the same adsorption column can be used for more than a month.

In an attempt to increase the adsorbing power of the sulphide, several factors have been studied.

(*a*) *Ageing of the precipitate.* If the precipitate is washed and activated immediately after it is prepared, the adsorbing power is approximately doubled, but does not show the same selectivity. It becomes impossible to redissolve certain amino-acids which are not precipitated by silver in aqueous solution, e.g. leucine. As far as we are aware, this is the first time that the phenomenon of the ageing of silver sulphide has been pointed out.

(*b*) *Optimum time of activation.* 12·5 g. of sulphide, activated for a few minutes only, retain

0·8–0·9 mg. of histidine; after 3 hr. 1·38 mg., and after 16 hr. 1·43 mg. Thus, the activation is practically complete after 3 hr. This rather long time may be due to diffusion of traces of H_2S occluded, or to a slow displacement of the silver ions adsorbed towards sites which are less accessible.

(*c*) *Modification of the state of aggregation.* No change of adsorbing power is obtained by grinding the previously dried precipitate (temp. $\leqslant 60°$) in an agate mortar till it passes through a sieve of 20 μ. Much more extensive pulverization is obtained by flattening between two polished glass plates, without previous desiccation, yet the activity is only doubled and the same diminution of selectivity is observed as in the case of the unaged precipitate.

(*d*) *Modification of the state of the surface.* It has been possible to double the adsorbing power without influencing the selectivity, merely by activating the Ag_2S with 0·1 M-HNO_3 for several hours, and then washing with 0·1 M-acetic acid. Negative groupings S_nO_m form on the surface, giving rise to new active centres after displacement by the acetate ions. The corrosive action of the nitric acid increases the heterogeneity of the surface. The question of augmenting the adsorbent power of mineral precipitates still has very numerous experimental possibilities and requires further study.

Behaviour of the different natural amino-acids

The adsorption of individual amino-acids has been studied, and where it occurs, the quantity adsorbed by 12·5 g. of sulphide and the stability of the adsorption compound in a more or less acid medium have been ascertained. Threonine and hydroxyproline are the only acids not studied. It is supposed that their behaviour is identical with that of the corresponding non-hydroxylated amino-acids because of their chemical relationship, and because the silver salts of these two acids have not been described and therefore very probably are soluble.

A few mg. of amino-acid are filtered through the column, and the excess eliminated by washing with 200 ml. of boiled distilled water (three portions, 0–100, 100–150, 150–200 ml.). Then, according to circumstances, a more or less acid solution is filtered through (0·001 M-acetate buffer of pH 5, 0·1 M or M-acetic acid) and the speed of elution determined by tests on successive portions of the filtrate.

In general, an excellent parallel has been observed between the adsorbability and the corresponding insolubility of the silver salt. Methionine is the only exception; although its silver salt is very soluble it is very strongly adsorbed to the extent of 0·5–1 mg. The stability differences of the adsorption compounds as a function of pH allow us to classify the natural amino-acids in four groups:

Group 4. This comprises the amino-acids not adsorbed at pH 6–7. They are glycine, alanine, serine, valine, threonine, leucine, proline, hydroxyproline, lysine and arginine. The results obtained with leucine proper (amino-*iso*-caproic acid) are probably equally valid in the case of *iso*leucine and *nor*leucine. 100 ml. water are sufficient to wash these acids into the filtrate, except in the case of leucine and of lysine, for which in practice 150 ml. are necessary for complete elimination.

Group 3. This consists of the acids adsorbed at pH 6, with easy elution; they are, in order of decreasing adsorbability, glutamic and aspartic acids, tryptophan, histidine, phenylalanine and tyrosine. The amounts of phenylalanine and tyrosine adsorbed are distinctly less than those of the other amino-acids (0·4 and 0·3 mg. respectively). Different

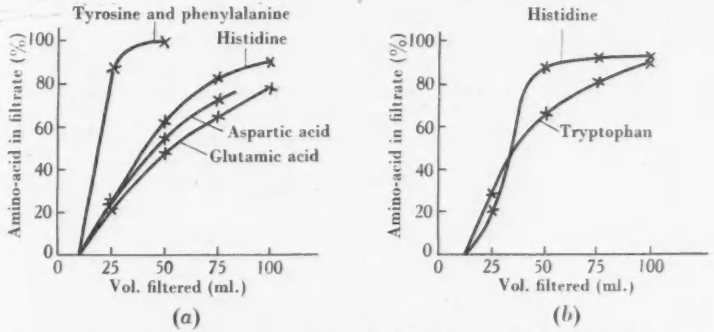


Fig. 2. Rate of elution of different amino-acids adsorbed on 12·5 g. Ag_2S by 0·001 M-acetate at pH 5, (*a*) from a column packed with the help of a pestle, (*b*) packed by suction.

factors can influence these adsorption phenomena: (*a*) The pH of the medium. Fig. 2, showing the elution speeds of these acids with a solution of 0·001 M-acetate buffer at pH 5, indicates that it should be possible to separate phenylalanine and tyrosine from the other amino-acids in this way. The difference in behaviour of histidine in the two

elution curves is due to the greater homogeneity of the column packed by suction. The reversal of the elution speeds shown in (*b*) is probably due to the buffering power of the histidine. (*b*) The presence of salts in solution. Any salt containing an anion whose silver salt is not very soluble is capable of modifying the adsorption equilibrium of the acids, to act, that is, as an eluting agent. The same column saturated with tyrosine, histidine and tryptophan was washed with 0·001 M solutions of Na acetate and Na_2SO_4. The elution of the tyrosine is complete after washing with 150 ml. of sodium sulphate, whilst 75 % of tryptophan and 25 % of histidine pass into the filtrate. The elution of the tyrosine is also complete after washing with 200 ml. of 0·001 M-sodium acetate. The reversal of the order of stability observed for histidine and tryptophan is due to the fact that the silver salt of the imidazole group is much more stable to SO_4 ions than to H ions. (*c*) The nature of the displaced ion. The stability of the adsorption compound of tyrosine is so slight that the acid passes into solution in the first 100 ml. of washing water, even when the amount involved is only 0·1 mg. To make the retention quantitative, it is enough to treat the column with 0·01 M solutions of soda and then of nitric acid, so that the NO_3 ions are substituted for the acetate ions retained electrostatically by the silver ions adsorbed (the value of 0·3 mg. given above relates to a 'nitric' column, all the others to 'acetic' columns). This change does not modify the behaviour of the column with respect to the acids of group 4.

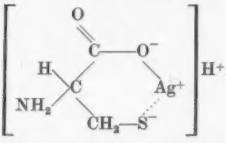
Group 2. Methionine, which constitutes this group, differs from the acids of the previous group by the distinctly greater stability of its adsorption compound at acid pH. The elution of glutamic acid is complete after washing with 50 ml. of 0·1 M-acetic acid. Table 1, on the other hand, shows the result of filtration of 0·4 mg. of methionine and washing with 0·1 M-acetic acid.

Table 1. *Speed of elution of methionine adsorbed on* Ag_2S *by* 0·1 M-*acetic acid*

Cut (ml.)	0–40	40–70	70–100	100–150	150–200
Methionine content (% of the amount put on)	0	0	24	25	21

This acid is recovered quantitatively by washing the column with 150 ml. of M-acetic acid. We are concerned here with a different type of adsorption due to the presence in the molecule of a very polarizable atom. It is the first example of a general method for isolating the sulphur-containing and halogenated organic compounds by adsorption in aqueous solution on insoluble sulphides. The carrying down of methionine by a precipitate of Cu_2O (Lucas & Beveridge, 1940; Bailey, Chibnall, Rees & Williams, 1943) may have the same origin. It is suggested that our results should be compared with those of these authors.

Group 1. There remain cystine and cysteine, which are adsorbed in the presence of M-acetic acid and, so far, have not been recovered by elution. In this case the type of adsorption appears different; the amounts adsorbed are about 3 mg., one part being irreversibly fixed, while the other is slowly eliminated by washing with solutions of 0·001 or 0·01 M-acetic acid, for example. If the amount of cystine or cysteine put on is only 1 mg., no amino-N or SH grouping (nitroprusside reaction) appears in solution on washing with N-acetic acid, N-H_2SO_4 or 0·04 M-ammonia, in spite of the solubility of their silver salts under these conditions. It does not seem probable that we are here concerned with the type of adsorption shown by methionine. Probably the following soluble complex $(C_3H_5NSO_2Ag)H$, which is very stable, forms on the surface. The structural formula suggested for this complex is



The precipitate of silver cysteinate may be the silver salt of this acid. This would explain not only our adsorption experiments, but also the behaviour of cystine and cysteine with silver salts in aqueous solution (Vickery & Leavenworth, 1930).

Quantitative separation of mixtures of natural amino-acids

A mixture containing 10 mg. each of ten natural amino-acids, i.e. the acids of group 3, excluding phenylalanine, and the following five acids of group 4: alanine, serine, valine, leucine and arginine, were dissolved in water, the pH adjusted to 6, and the solution diluted to 200 ml. 57·7 % of the amino-N should pass into the filtrate.

If an attempt is made to separate the mixture on an 'acetic' column, tyrosine passes into solution; the filtrate obtained by introducing into the column only the five amino-acids of Group 3 gives a positive diazo reaction, and in the case of the mixture of the ten acids, about 65 % of the amino-N passes into the filtrate. The tyrosine is held in so labile a way that even a considerable diminution of the amount of amino-N fixed (down to 42 % of the amount of histidine adsorbed by the column) does not appreciably improve the retention. A 'nitric' column must be used. The separation is then quantitative, except, obviously, when the limit of saturation of the column is exceeded. Thus the mixture of the five acids of Group 3, corresponding to 0·156 mg. of

amino-N were put on 25 g. of sulphide previously activated by 0·1 M-HNO_3; the cuts 0–150, 150–250 and 250–350 ml. gave completely negative diazo reactions and the elution was practically quantitative after washing with 200 ml. of 0·1 M-acetic acid. Then, on the same column, the mixture of the ten amino-acids corresponding to (*a*) 0·27 mg., and (*b*) to (*e*) 0·294 mg. of absorbed amino-N were run through. The following results were obtained (Table 2).

Table 2. *Quantitative separation of a mixture of ten natural amino-acids by adsorption on* Ag_2S

(The quantitative separation corresponds to a passage of 57·7 % of amino-N into the filtrate.)

		Filtrate amino-N		Eluate amino-N	
Exp.	Fraction	mg.	% of amino-N put on	mg.	% of amino-N put on
(*a*)	0–200	0·1540	57·1	0·114	42·2
	200–250	0·0030	1·0	0·004	1·7
	250–300	0·0000	0·0	0·003	1·2
	300–500	0·0000	0·0	—	—
			58·1		45·1
		Total		103·2 %	
(*b*)	0–200	0·1515	56·2	0·111	41·1
(*c*)	0–200	0·1585	58·7	—	—
(*d*)	0–200	0·1760	60·0	0·1190	40·5
	200–250	0·0010	0·5	—	—
			60·5		40·5
		Total		101·0 %	
(*e*)	0–200	0·1800	61·0	0·1050	36·0
		Total		97·0 %	

The amount put on in Exps. (*d*) and (*e*) is higher than the saturation limit; diminishing the amount used again permitted quantitative retention to occur (negative diazo reaction). In practice, the quantity used will be kept below the saturation limit of the column, determined preferably with the acid the least strongly adsorbed; alternatively, the quantitative character of the separation obtained will be controlled by a colour reaction or a second experiment on a new column. It seems unnecessary to give further similar examples of analysis. We confine ourselves only to drawing attention to the fact that the modification of the adsorption by the influence of pH, of salts and of the nature of the displaced ion provides methods of separation with manifold possibilities applicable to any mineral precipitate.

DISCUSSION

Protein hydrolysates can be quantitatively separated by chromatography on precipitated silver sulphide. If the weak adsorbing power of the precipitate makes this technique less convenient in actual fact than other chromatographic methods, on the other hand it allows separations to be made which are very different from those so far obtained: quantitative isolation of methionine almost directly from a protein hydrolysate, and separation, in the same sample, of amino-acids differing as much as those in Group 3. Because of its originality it can be used for the analysis of proteins and for contributing to the solution of microbiochemical problems.

But the results just described are of interest from the more general point of view of chromatography in aqueous solution. They show that this technique can be applied to silver sulphide and that this precipitate behaves in an exactly similar way to that of an 'acid' column of alumina. There is the same irreversibility of the adsorption (histidine, etc.), the same influence of the displaced ion and the existence of the same elution phenomena. But silver sulphide differs from the other adsorbents, in that it forms well-defined adsorption compounds whose solubility properties are well known. This permits of predictable separations, and the *a priori* determination of adsorption and elution conditions of the different constituents of a mixture. (The exceptions themselves are easily explained, as we have seen for S-containing amino-acids.) As the adsorbent properties of the sulphide depend only on its insolubility (which determines the formation of a precipitate which has a large surface and implies a high adsorption energy of the ions of the lattice), it can be foreseen that the other mineral precipitates, and particularly those which are least soluble, will behave in the same way. This is completely confirmed by similar laboratory investigations with a precipitate of barium sulphate (Tarte, 1945). This generalization puts at the disposal of investigators a series of new adsorbents which have very numerous possibilities (formation by adsorption of insoluble salts, of complexes, and of addition compounds stable in aqueous solution). Chromatography in aqueous solution should be profoundly modified by giving it a more deductive character. It shows indeed that this technique, which so far is so arbitrary and empirical, should be systematized.

Further, our investigations may permit a better understanding of the phenomena of the adsorption of organic compounds on the surface of mineral precipitates in aqueous solution. In particular they can throw light on the amino-acids that are selectively lost when mineral precipitates are discarded during a protein analysis, and suggest methods for controlling or preventing these losses.

SUMMARY

1. The use of sparingly soluble mineral precipitates, commonly available in the laboratory, as adsorbents for chromatography in aqueous solution, is suggested.

2. A flocculate of silver sulphide, left for several hours in contact with a dilute solution of silver nitrate, acquires considerable and highly selective adsorbent properties.

3. A new quantitative method for separating natural amino-acids is based on these properties. Different types of adsorption are observed which can be regarded as examples of more general phenomena which occur on the surface of mineral precipitates.

4. These investigations indicate new preparatory and analytical possibilities in the sphere of microbiochemistry and suggest methods for eliminating the adsorption of organic compounds at the surface of mineral precipitates, a phenomenon which so often invalidates the determinations of these compounds.

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An Artificial Standard for Use in the Estimation of Haemoglobin

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Colorimetric procedures for determining haemoglobin have the disadvantage that they must normally be standardized by oxygen capacity or iron determinations on blood, and this process must be repeated at intervals to guard against deterioration of the standard. To avoid the necessity for these measurements, artificial standard solutions have been proposed for some of the commoner methods of estimating haemoglobin. They have not been widely adopted because they are suitable only for use in visual colorimeters under definite conditions of illumination. The absorption spectra of the

standards have usually been so widely different from those of the blood derivatives that any marked variation in the quality of the lighting of the colorimeter has been inadmissible, and they are not suitable for use in modern absorptiometers working with approximately monochromatic light.

The introduction of haemin as a standard substance (Rimington, 1941; Clegg & King, 1942) offered a prospect of considerable improvement in some of these respects. The latter authors stated that haemin could be weighed out to give a stable solution in dilute alkali, that the intensity of colour of the solution was proportional to the iron content of the haemin sample used to prepare it, and that the absorption spectrum of the standard solution was similar to that of blood converted to alkaline haematin by heating with dilute alkali.

During the course of work involving the accurate estimation of haemoglobin in many blood samples, we were impressed by the convenience and reproducibility of results obtained by the alkaline haematin method described by Clegg & King (1942). We have found, however, that some samples of haemin give a colour much in excess of that to be expected from their iron content, that the solutions change appreciably with time, and that there are significant differences in absorption spectrum between individual samples of haemin.

The principal qualities of a satisfactory standard for general use in haemoglobinometry are that it should be reproducible, stable, easily prepared, and have an absorption spectrum closely similar to that of the blood derivative with which it is to be compared. We have prepared a standard solution with certain inorganic salts which can readily be obtained in a pure form. It is stable, and, over a considerable range of wave-lengths, reproduces closely the absorption spectrum of blood treated with dilute alkali. We have assigned a haemoglobin value to this solution by comparison with a number of blood samples, the haemoglobin content of which we determined by very careful estimations of oxygen capacity and iron content.

METHODS

Apparatus. All volumetric apparatus was calibrated. Pipettes used to deliver blood were calibrated by delivery of blood of known specific gravity. Weights were checked against a National Physical Laboratory standard set.

Blood samples. 50 ml. of venous samples were taken from normal male medical students, usually about 2–3 hr. after breakfast. A stainless needle was used, and coagulation prevented by an oxalate mixture (1 mg./ml. of blood), consisting of three parts ammonium oxalate and two parts potassium oxalate. The blood was mixed well and the portions required for the various analyses taken at once.

Oxygen capacity. Triplicate determinations were made by the method of Van Slyke & Neill (1924). Oxygen was not determined by absorption, but the total volume of extracted gases (other than CO_2) was measured, and corrected for O_2 and N_2 in physical solution according to Sendroy, Dillon & Van Slyke (1934) and Van Slyke, Dillon & Margaria (1934). The advantage of this method is that any carboxyhaemoglobin present will be included in the estimation. The average deviation of a determination from the mean was 0·05 g. of haemoglobin/100 ml. of blood. The apparatus was calibrated physically according to Peters & Van Slyke (1932), and chemically by the liberation of nitrogen from hydrazine by iodate. In two cases, CO capacity was measured by the method of Van Slyke & Hiller (1928) as an additional check.

Methaemoglobin. This was determined in all samples by the method of Evelyn & Malloy (1938) as modified by Barcroft, Gibson, Harrison & McMurray (1945). The blood sample was taken from the oxygenator during the O_2 capacity determinations, and the figures given for oxygen have all been corrected by the addition of an amount corresponding to the quantity of methaemoglobin present. In our samples this was never greater than 0·1 g./100 ml. This figure, however, does not necessarily represent the true level of methaemoglobin in normal blood, as reduction of the pigment would be likely to occur during oxygenation of the blood.

Determination of iron in blood. This was carried out on 3 ml. samples of blood by a slight modification of the method of Klumpp (1934). The blood samples were ashed in 25 ml. conical Pyrex flasks, instead of porcelain dishes. This has the advantage that the risk of contamination is reduced, that losses by sputtering are less likely, and that transference of the ash from one vessel to another is avoided. 0·3 ml. of iron-free conc. H_2SO_4 was added to each of the samples, which were then placed in a glass-lined oven at 105° for 4–6 hr. The flasks were then taken singly, supported on an inclined silica triangle under a glass screen in a fume cupboard, and heated with a Bunsen flame. The neck and upper part of the flask was heated first and the heating gradually extended downwards, thus causing the froth to break up. The heating was continued for a few minutes until most of the H_2SO_4 had been driven off and the contents of the flask have become solid. If this precaution is omitted and the flasks put straight into the muffle, many determinations are spoiled owing to the contents pouring over the neck of the flask. Ashing was completed overnight at about 520° in an electric muffle fitted with a removable silica lining, the flasks being placed nearly horizontally to facilitate escape of CO_2. When the residue was quite free from carbon, the sides of the flasks were washed down with 5 ml. 10% (v/v) H_2SO_4, and the ash dissolved by heating at 105° until the last traces of iron oxide had gone into solution. This is a slow process, requiring some hours. The contents of the flasks were then diluted to about 5 ml. with distilled water and heated for a further 20–30 min. to dissolve any $Fe_2(SO_4)_3$ which might have separated out. The titration was completed, after thorough cooling of the flasks, exactly as described by Klumpp (1934). A control flask containing the reagents (including the anticoagulant used for the blood) was always carried through all the stages of the method. The determinations were done in duplicate or triplicate. The average deviation from the means was 0·5% of the amount measured. The blood haemoglobin was calculated on the assumption that haemoglobin contains 0·334% of Fe. In a few cases the values obtained by

$TiCl_3$ were checked by a dichromate method based on that of Bernhart & Skeggs (1943).

Determination of iron in haemin. Samples of about 40 mg. were weighed out and treated with 0·3 ml. of conc. H_2SO_4. In this case, preliminary heating was unnecessary, and the samples were placed directly in the muffle. The final volume was 6 ml., and 6 ml. of standard iron solution were used. Otherwise the procedure was the same in the two cases.

Preparation of iron standard solutions. Strong standard solutions were prepared (i) by dissolving electrolytic iron ('H.S.' brand (Johnson, Matthey & Co.), better than 99·9% pure) in 10% (v/v) H_2SO_4, with precautions to avoid loss as spray, and making up to a volume such that the concentration was about 5 mg. of Fe/ml.; and (ii) by dissolving ferric alum in 2 N-H_2SO_4. The alum was not regarded as a standard substance. Iron was then determined in these solutions by dichromate titration following reduction of ferric iron by $SnCl_2$, using N-phenylanthranilic acid as indicator. Agreement to within 1 in 1000 was secured between the results of the dichromate titrations and the expected values based on the weight of pure iron taken. Dilute standard solutions were prepared from the iron alum by dilution (1 in 10) with 2 N-H_2SO_4. The ferrous iron solutions resulting from the use of the pure iron required preliminary oxidation before dilution. In our hands, the use of $KMnO_4$ oxidation immediately before titanium titration has not led to consistent results. Oxidation was carried out by two methods: (*a*) 25 ml. of strong standard were made alkaline with NH_4OH, and iron-free H_2O_2 added. The mixture was left overnight and boiled for 10 min. to destroy any residual H_2O_2. The precipitate was then dissolved in 2 N-H_2SO_4 and made up to volume. (*b*) As a check on the first method of preparation, a dilute standard was prepared by oxidizing 25 ml. of solution with 1 ml. each of conc. HNO_3 and conc. H_2SO_4 in a Pyrex Kjeldahl flask, heating being continued to dryness. The residue was then taken up in conc. H_2SO_4, diluted, cooled, and made up to volume. Dilute standards prepared by the different methods agreed exactly on titration with $TiCl_3$. In the determinations on blood, the volume of standard used was such that the amount of iron contained was roughly similar to that in the blood sample.

Spectrophotometric measurements. In order to obtain approximate absorption curves of the salts used in preparing the artificial standard, the spectra were photographed on Ilford Process Panchromatic plates with a Hilger medium quartz spectrograph and sector photometer. The plates were read with a Hilger microphotometer. In making detailed comparisons between the form of absorption curves of blood solutions and of mixtures of the salts, the spectra were photographed with several different times of exposure and the densities compared with a series of standard densities prepared by photographing the spectrum of the light source on the same plate with varying times of exposure. The light source was a tungsten filament run by storage cells of large capacity.

Measurements in photoelectric absorptiometer. The instrument used was the original type of Hilger Spekker absorptiometer, with the addition of an external mirror galvanometer which permitted the use of relatively dense filters transmitting over narrow ranges of wave-lengths. Measurements were carried out with 1 cm. cells, carefully matched against one another. Variability of the absorptiometer readings was controlled by the use of suitable Ilford neutral density screens which were always read in front of the solvent cell immediately after each solution.

Use of filters. In routine use of the absorptiometer, it is a matter of considerable practical convenience if the graph relating optical density and haemoglobin concentration is linear and passes through the origin, since it may then be replaced by the use of a factor. This point may readily be checked by taking readings of serial dilutions of a blood converted to alkaline haematin. It is desirable to determine if this is the case for any particular filter used, since the results are modified both by the transmission curve of the filter and the spectral response of the photo-cells. In particular, we have found that certain filters did not give a straight line in our instrument because of transparency in the near infra-red (e.g. Wratten 62, Ilford Hg green). We have found the following filters satisfactory from this point of view: Chance OGr. 1, 2 mm., i.e. Hilger H 455, no. 5 (maximum at 530 mμ), and (with Hilger H 503 2 mm., infra-red absorbing glass) Ilford Spectrum filters nos. 603–606 inclusive (maxima at 490, 520, 550 and 580 mμ respectively). For visual colorimetry, any yellow-green or green filter may safely be used.

Preparation of blood for absorptiometer. 1 ml. blood from an Ostwald pipette was added to 100 ml. of 0·1 N-NaOH in a glass-stoppered volumetric flask. After repeatedly inverting for a few minutes until the solution was perfectly clear, about 10 ml. were poured into a glass-stoppered boiling tube, heated for exactly 4 min. in a vigorously boiling water-bath and cooled immediately by immersing in cold water (Clegg & King, 1942). At the same time, 10 ml. of the standard solution were heated and cooled in exactly the same way.

Preparation of artificial standard

Precise method of preparation

Potassium dichromate. Dry about 5 g. of $K_2Cr_2O_7$ (Analar), in an oven at 100° or above. Weigh out 0·690 g. and dissolve in about 80 ml. of distilled water.

Chromium potassium sulphate. Weigh out 3·42 g. of $K_2Cr_2O_7$, dissolve in about 100 ml. of distilled water in a 500 ml. conical flask, pipette in 25 ml. N-H_2SO_4 (accurately prepared) and then add slowly, from a measuring cylinder, a freshly prepared saturated solution of SO_2, until reduction of the dichromate is complete, i.e. when the solution becomes pure green, free from any trace of yellow. Then add a further equal volume of SO_2 solution to ensure excess. A total of 50–60 ml. of SO_2 solution is usually sufficient. It is most conveniently prepared from a siphon of liquid SO_2.) Add a few glass beads to prevent bumping, and boil briskly for 45 min. or more until all traces of SO_2 have been removed and the volume is considerably reduced. A wide-stemmed funnel should be placed in the neck of the flask to prevent loss by splashing. Dilute to about 200 ml. and allow to cool.

Cobaltous sulphate. Heat about 30 g. of $CoSO_4.7H_2O$ (Analar) in a small porcelain dish for about 2 hr. in an oven just below its m.p. (96°). Stir frequently with a glass pestle or the bottom of a wide glass test-tube and break up the coarser particles. Then heat overnight at about 400° in an electric muffle. Transfer the product, which should be a uniform lilac powder, while still hot, to a stoppered bottle, and when it is cool, weigh out 13·10 g. The substance is hygroscopic, and this weighing should be done as quickly

as possible. Dissolve in about 80 ml. of distilled water with the aid of heat.

If a muffle with pyrometer is not available, the salt may be prepared by drying at less than 96° as above, then covering the dish with another similar one and placing on a loose pad of asbestos about 1 cm. thick between two larger porcelain dishes which are then directly heated by a fairly strong Bunsen flame for several hours. The salt should be stirred and coarse particles broken up at intervals. A thermometer placed in the salt should read at least 300°, but the heat must not be strong enough to cause local blackening. Alternatively the water content may be determined on a sample of the hydrated salt, and an equivalent amount of this weighed out.

To complete the preparation of the standard, transfer the solutions of $CoSO_4$ and $K_2Cr_2O_7$ quantitatively to the flask containing the chromium, and transfer the mixture to a litre volumetric flask, washing out the conical flask and making up to the mark with distilled water.

Simplified method of preparation

Weigh out 11·61 g. of $CrK(SO_4)_2.12H_2O$ (Analar), using crystals free from any signs of whitening due to efflorescence, 13·10 g. of anhydrous $CoSO_4$, prepared as above, and 0·690 g. of $K_2Cr_2O_7$; dissolve in about 500 ml. of distilled water, add 1·8 ml. of N-H_2SO_4, and heat to boiling. Boil for 1 min., cool well and make up to 1 l. with distilled water. Several preparations have been made by this method, and all have given readings in the absorptiometer in close agreement with those given by the standards made by the precise method.

Notes. (i) In the freshly prepared standard (precise method), the ratio of the two forms of chromium sulphate is different from that in the standard as used. The standard is relatively greener, and on standing for several weeks, becomes reddish. Heating for 4 min. in a boiling water-bath before use is always necessary to obtain the correct equilibrium between the two forms of chromium sulphate.

(ii) The standard should be stored in a glass-stoppered bottle free from grease and organic material. Ethanol or acetone should not be used for drying tubes and bottles used to contain the standard.

(iii) The amount of acid has been defined precisely since the green chromium sulphate is considerably hydrolyzed and large changes in the amount of acid would alter the colour. The pH of a freshly heated and cooled solution is 2·15 at 18°, most of the acid coming from hydrolysis.

(iv) The indirect method of preparation of chromium potassium sulphate has been used because it is not easy to prepare the anhydrous salt in soluble form or to determine the water content of samples of the alum without risk of loss of SO_3.

RESULTS

Haemin as a standard substance in haemoglobinometry

Iron content and light absorption. Clegg & King (1942) found that the extinction of solutions of crystalline haemin in 0·1 N-NaOH was proportional to their iron content. In the thirteen samples examined by them, the coefficient of variation of the specific extinction was 1·18 %. Their measurements were made with a photoelectric absorptiometer with a Chance green filter, and with a visual colorimeter with Hg green illumination. We have repeated these observations on six samples of haemin, and have found that in two of them there was a substantial discrepancy between iron content and light absorption with the Chance green filter. The results are shown in Table 1. While certain of

Table 1. *Iron content and optical density of haemin samples*

(The specific extinction, E_s, is the optical density of a solution containing 1 mg. of haemin Fe in 100 ml. of 0·1 N-NaOH solution, measured in a 1 cm. cell with a 2 mm. OGr. 1 filter (Chance) in the absorptiometer.)

Haemin sample	Description	Fe content (%)	E_s	Deviation from average (%)
A	Crystalline	7·97	0·776	−3·4
B	Amorphous	8·18	0·815	+1·5
C	Crystalline	8·29	0·786	−2·1
D	Crystalline	8·23	0·792	−1·4
E	Amorphous	8·00	0·865	+7·8
F	Crystalline	8·17	0·784	−2·2
			Average	0·803±3·1

E was a sample of haemin issued by a commercial firm for the purpose of haemoglobin standardization. The other samples were made by workers in several different laboratories.

the samples behave as predicted by Clegg & King, errors of up to 8 % may be involved in the acceptance of iron content as a basis for the use of haemin as a standard for haemoglobinometry.

Light absorption of blood as alkaline haematin and of haemin solutions in different spectral regions. If a standard for haemoglobinometry is to be suitable for use with a variety of filters, its absorption spectrum must be similar to that of the blood derivative with which it is to be compared. We have investigated this point by measuring the extinction of haemin solutions and of blood, both in 0·1 N-NaOH, with the absorptiometer over the range covered by the Ilford spectrum filters nos. 603–606 inclusive. Using ten samples of normal blood, we measured the extinction with each filter and calculated the average ratios of extinctions with the different filters as compared with the Chance green filter OGr. 1. We carried out a similar series of measurements with solutions of each of the haemin specimens and similarly calculated the ratios of the extinctions with the different filters. In general, the ratios with the haemin solutions differed considerably among themselves and differed also from those obtained with blood. Arbitrarily taking the haemoglobin equivalent of each haemin solution with the OGr. 1 filter as 15·6 g. Hb/100 ml. and comparing the filter ratios from blood with those

from each haematin specimen, we calculated the haemoglobin equivalent of each haematin solution for all the other filters. The results are shown in Table 2. It is clear that the absorption spectrum of

Table 2. *Haemoglobin equivalents of haemin solutions with various colour filters*

Haemin sample	Ilford filter no. Y 606 (g. of haemoglobin/100 ml.)	YG 605	G 604	BG 603
A	14·5	14·8	16·8	15·8
B	15·1	15·4	16·1	15·9
C	14·7	15·1	16·8	15·8
D	15·6	15·4	16·7	16·2
E	15·6	15·1	16·1	16·8
F	15·1	15·3	16·5	16·1
Artificial standard	15·7	15·4	15·6	15·7
Blood	15·6	15·6	15·6	15·6

The figures give the haemoglobin equivalents of each haemin solution with the various filters, taking the haemoglobin equivalent with the OGr. 1 (2 mm.) Chance filter as 15·6 in each case.

haemin differs appreciably from that of blood, and that individual haemins differ from one another. It follows that errors will be introduced by changes in the colour of light used in the measurements, and these are additional to those which may arise through lack of proportionality between colour and iron content. It should be noted that these comparisons were made on haemin solutions prepared only 24 hr. previously, for we have found that haemin solutions change markedly in colour and absorption spectrum on prolonged storage, even in the dark. Visual inspection of most haemin solutions and of blood treated with alkali showed an obvious difference in colour, the blood being greenish, the haemin reddish brown with a slightly violet tinge. Solutions of the two amorphous samples of haemin (B and E in Table 1), however, had a colour very closely similar to that given by blood. On keeping for some time, the brown colour of the haemin solutions gradually changed to the greenish colour given by blood.

The development of an artificial standard

It is clear that the use of haemin as a standard substance for haemoglobinometry may lead to considerable errors. We therefore investigated the possibility of preparing a standard solution from a mixture of inorganic salts. Such a standard, we hoped, would at least have the advantages of reproducibility and stability. Since the more accurate colorimetric and absorptiometric methods for estimating haemoglobin usually involve measurements with filtered light of a fairly narrow range of wavelengths, we aimed at the preparation of a mixture whose spectral absorption was similar to that of blood treated with alkali over a considerable portion of the visible spectrum, regardless of whether or not there was also a good visual match. We prepared rough absorption curves for various salts to aid us in choosing the components for our standard, and used, finally, chromium sulphate in its green modification (giving a band similar to the α-band of alkaline haematin), together with cobaltous sulphate (supplying a broad band filling the gap between the bands of chromium sulphate in the yellow and blue), and a small quantity of potassium dichromate to increase the absorption in the blue-green. The exact amounts of the components were settled by trial. The closeness of fit finally achieved may be indicated by the fact that if the haemoglobin equivalent with a 2 mm. Chance OGr. 1 filter be taken as 100, the values with Ilford filters were: no. 603, 100·8, no. 604, 99·8, no. 605, 98·7, no. 606, 100·8. These results compare very favourably with those obtained with haemin (cf. Table 2).

Some properties of the artificial standard

Reproducibility. As the method of preparation of the standard involves the weighing out of relatively large amounts of two substances, one of which, $K_2Cr_2O_7$, is a primary analytical standard, and the other, $CoSO_4$, is used for weighing in the gravimetric analysis of cobalt, it might be expected that the reproducibility of the standard would be good. This point has been confirmed by the preparation of eight samples of standard from different specimens of the reagents.

A difficulty arises in connexion with the reproducibility of the standard since, in solution, the two modifications of chromium sulphate, green and violet, exist in equilibrium with one another. At low temperatures, the equilibrium mixture contains considerable amounts of the violet salt, but at 100° the green form predominates. It has been found that a definite equilibrium is reached very rapidly at 100°. In practice, 4 min. on a boiling water-bath is sufficient. Heating the standard, besides being essential for this reason, has the advantage that, since the blood must be heated for a similar period in the method of Clegg & King (1942), possible losses from evaporation will be compensated.

Stability. (*a*) *Immediate stability.* After the standard has been heated and cooled, the green and violet forms of chromium sulphate are no longer in equilibrium, and on standing, the amount of the violet form increases slowly. We find that at room temperature the change in absorption in the green region is only about 1 % in the first hour, and after that the rate of change is considerably less. (The total diminution in absorption after prolonged standing is of the order of 10 %.) It is quite safe, therefore, to use the standard within ½–1 hr. of

heating. We recommend that a fresh portion of standard be taken on each occasion to eliminate the risk of cumulative errors due to evaporation during the heating.

(*b*) *Long-term stability.* We have found that the following treatments do not cause changes exceeding 1%: (*a*) standing under ordinary laboratory conditions for 8 months; (*b*) exposure for 6 hr. in a quartz tube to the light of a quartz Hg vapour lamp at a distance of 10 in.; (*c*) south exposure in a quartz tube to outdoor conditions for 3 months; (*d*) heating in a sealed tube at 95–101° for 48 hr. (heating to temperatures above 105° caused formation of a precipitate); (*e*) boiling down six times to one-fifth of initial bulk and making up to original volume. In view of the resistance of the standard to these adverse conditions, we consider that it may be expected to remain stable indefinitely under ordinary laboratory conditions.

Beer's Law. It is desirable that the standard should, on dilution, behave in the same way as blood, and that the same haemoglobin value be obtained for a given blood when several dilutions of the blood are compared with several dilutions of the standard. To test this point, blood was diluted with various amounts of 0·1 N-NaOH, up to four times the usual amount. The resulting solutions were heated and cooled as usual and compared with similar dilutions of the standard made, after heating and cooling, with distilled water. The solutions were compared, each one with all the others, in the absorptiometer with the OGr. 1, and Ilford Spectrum filters nos. 603–606 inclusive with Hilger H. 503. The results indicated that, within the range examined, Beer's Law is obeyed both by the solution and by blood. The standard may be used for direct comparison with samples of blood having haemoglobin values widely different from that of the standard. If it be preferred, however, a diluted standard may be prepared for use with bloods low in haemoglobin by addition of distilled water to the standard after heating and cooling.

Haemoglobin equivalent of the standard

To determine this figure, we have measured the light absorption of a number of samples of normal blood of known haemoglobin content, diluted 1 in 101 and treated by the method of Clegg & King (1942). The results have been compared with those given by the standard.

These measurements have been carried out in the absorptiometer with Ilford Spectrum filters nos. 603–606 inclusive, and with the Chance green, OGr. 1. We have also made comparisons in the visual colorimeter, in natural and artificial light, both with and without filters.

The haemoglobin values of the blood samples in the series were determined by measurement of both O_2 capacity and iron content. Table 3 shows the figures obtained for the haemoglobin equivalent of the standard. The average value for the coefficient of variation is 1·6% derived from O_2 capacity and 1·1% from Fe. These values indicate a satisfactory parallelism between O_2 and colour and between Fe and colour. Although this variation is small, we consider that the differences between individual bloods lie outside the range of experimental error. In both series the Spectrum Blue-Green filter (Ilford 603) gave rather less consistent results than the other filters. When the value of the standard was being determined, we standardized an Ilford Neutral Grey Screen over the same series of bloods, with the OGr. 1 filter only. In this case the coefficient of variation from oxygen was 0·6% and from iron 0·5%.

Table 3. *Haemoglobin equivalents of standard*

(g. of haemoglobin/100 ml.)

	From oxygen capacity					From iron determination				
Filters ...	OGr. 1	606	605	604	603	OGr. 1	606	605	604	603
Blood no.										
1	15·7	15·6	15·4	15·5	15·5	16·1	16·0	15·8	15·9	15·9
2	15·5	15·5	15·2	15·5	15·5	15·9	15·9	15·6	15·9	15·9
3	15·4	15·6	15·3	15·5	15·6	15·9	16·1	15·8	16·0	16·1
4	15·5	15·4	15·2	15·3	15·5	16·1	16·0	15·8	15·9	16·1
5	15·8	16·0	15·6	15·8	16·3	16·1	16·3	15·9	16·1	16·6
6	16·1	16·2	15·8	16·0	16·2	16·4	16·5	16·1	16·3	16·5
7	15·5	15·8	15·3	15·4	15·5	16·0	16·3	15·8	15·9	16·0
8	15·6	15·6	15·4	15·5	15·8	16·0	16·0	15·8	15·9	16·2
9	15·8	15·8	15·4	15·7	15·8	16·1	16·1	15·7	16·0	16·1
10	15·4	15·6	15·3	15·5	15·5	15·8	16·0	15·7	15·9	15·9
Averages	15·6	15·7	15·4	15·6	15·7	16·0	16·1	15·8	16·0	16·1
	Average from O_2 = 15·60					Average from Fe = 16·01				

The figures represent the haemoglobin equivalent of the standard calculated on the assumption that 1 g. Hb combines with 1·34 ml. O_2, and contains 0·334% Fe.

The use of the artificial standard in visual colorimetry was investigated with a further series of ten normal blood samples. These were diluted and heated with alkali in the ordinary way and read against the standard in two colorimeters, illuminated by daylight and artificial light without filters and with a variety of green filters (Chance OGr. 1, Wratten 62, Leitz green (as supplied with colorimeter), and Ilford 604). At the same time, the haemoglobin values were determined against the standard in the absorptiometer. The results with the visual colorimeters agreed closely in each case with those obtained in the absorptiometer, indicating that the standard is suitable for visual colorimetry.

Although the tint of the standard is not absolutely identical with that of blood treated with NaOH (it appears very slightly greener when freshly heated), yet it is so nearly the same that we found no difficulty in matching with unfiltered light. The haemoglobin equivalent with unfiltered light is slightly higher than that found with filters, owing to deviations in the absorption spectrum of the standard in the red and violet, and should be taken as 16·2 g./100 ml. on an iron basis.

The effect of temperature on the haemoglobin value of the standard

The optical density of the standard depends to a small extent on its temperature. The average values given in Table 3 for the haemoglobin equivalent are valid at 18°, and, for ordinary purposes, the effect of temperature may be disregarded. For accurate work, however, the values given in Table 3 should be corrected by adding the following quantities in g. of haemoglobin/100 ml. for each degree by which the temperature of the standard exceeds 18°. Filters: OGr. 1, 0·03; Ilford 606, 0·015; Ilford 605, 0·03; Ilford 604, 0·04; Ilford 603, 0·02. If the temperature of the standard is less than 18°, corresponding amounts should be subtracted. (Although the relation between density and temperature is not strictly linear, the error introduced is quite negligible.) The temperature coefficient of blood in 0·1 N-NaOH is relatively small and may be neglected.

Application of standard to routine determinations of blood haemoglobin

Pipette 10 ml. of a clear solution of 0·1 N-NaOH into a dry boiling-tube, about 2·5 × 15 cm., fitted with a loosely fitting ground-glass stopper. This may be made in a few minutes by grinding a stopper into an ordinary boiling-tube with moderately coarse carborundum. Ordinary corks must not be used as they give a colour with the alkali. Pure NaOH (Analar) should be used, and the solution should be stored in a Pyrex vessel. Some impure samples of NaOH give an opalescence when heated with blood. Add 0·1 ml. well-mixed blood from a calibrated pipette, stopper and mix gently for a few minutes until the solution is clear. Take about the same volume of standard in a similar tube and immerse the two tubes (with stoppers loosened) in a vigorously boiling water-bath so that the liquid in them is just covered by the water. Heat for exactly 4 min. At once cool thoroughly by immersing in cold water and mix by inversion after wiping the outside of the tubes.

Visual colorimeter. (*a*) *Used as colorimeter.* Set standard at 10 mm. and compare, preferably with a green filter over the eyepiece. Readings should be taken as quickly as possible before the eye becomes fatigued. Haemoglobin value for standard = 16·0 g./100 ml. using filters and 16·2 g./100 ml. using unfiltered light (both on an iron basis).

(*b*) *Used as a photometer.* Place a neutral screen of about 0·5 density on one side, and the standard solution on the other. View the fields through any sharply selective light filter (such as an Ilford spectrum filter or Hg green filter) transmitting maximally near the middle of the spectrum. A series of readings is taken, the average of which (*s* mm.) will correspond to 16·0 g. of haemoglobin/100 ml. on an iron basis. If now a blood solution is substituted for the standard, and gives a reading of *B* mm., the haemoglobin value of the blood will be $\frac{16{\cdot}0 \times S}{B}$ g./100 ml. For accurate work, the screen and solution should be used alternately on both sides of the colorimeter and the readings averaged. Once the haemoglobin value of the screen has been determined, the standard need be used only to check the screen at intervals.

Photoelectric absorptiometer. The standard and the blood solution are read alternately in similar 1 cm. cells against water, using the Chance OGr. 1 (identical with Hilger H 455, no. 5) or other suitable filter (see under Methods). It is convenient to use the standard to calibrate an Ilford neutral grey screen of about 0·5 density, as suggested by King, Gilchrist & Delory (1944). With the colour filter in position the standard is read against water and then the grey screen is read in front of the water cell. Having assigned a haemoglobin value to the screen by several comparisons with the standard, it may then be used with that particular filter at any time for comparison with different bloods, and a check-up with the standard need only be done occasionally. We would mention, in this connexion, that the density value marked on the filters cannot be taken as the reading to be expected on any particular absorptiometer, as its value depends on the optical conditions of measurement. It should be emphasized that the haemoglobin value assigned to the screen is only valid with the particular colour filter used for its standardization.

DISCUSSION

The consistent difference between the haemoglobin values calculated from Fe determinations and oxygen capacity measurements is not due to carboxyhaemoglobin or methaemoglobin, since both of these pigments were included in the figures for oxygen capacity. In view of the special care exercised in standardizing our iron and oxygen methods, and the consistent difference found with each blood, we consider the discrepancy a real one, indicating the presence of some 3 % of the iron in a non-functional form. It is not due to neglect of plasma iron, since the amount of this in normal

blood (one-eighth % of total iron; Fowweather, 1934) is too small to account for the difference. It is interesting that similar results have recently been obtained by Macfarlane & O'Brien (1944) on a series of samples of male blood.

The artificial standard has the disadvantages that it has a relatively large temperature coefficient and that it must be heated shortly before use. On the other hand, it has the following advantages over haemin as a standard: it is stable and reproducible, being made of inorganic substances easily obtained pure; it does not require the use of a very sensitive balance in its preparation; it gives a better colour match with alkaline solutions of blood and more closely follows the blood spectrum over the middle range of the visible spectrum; its use eliminates the need for the lengthy and difficult analysis of iron involved in the use of haemin. Our experience leads us to believe that the systematic error involved in using the standard as described should not exceed $\pm 1{\cdot}5\%$, i.e. $\pm 0{\cdot}2$ g. of haemoglobin/100 ml. in a normal blood. In view of the results of Mole (1945) on the variability of blood samples from the same individual, it would appear that no practical advantage would be gained from a greater degree of precision.

Although we have taken every care in determining the haemoglobin value of the standard, it is possible that further work may lead to a small revision of the figures given. In that case, any necessary correction could easily be applied to haemoglobin determinations already made by the method. We consider that the reproducibility of the standard is such that it should provide a useful basis for haemoglobin surveys carried out at different times or in different places.

It is hoped that the introduction of this standard will make relatively accurate haemoglobinometry possible in any ordinarily equipped laboratory. It may be pointed out that, in addition to its use in the routine estimation of haemoglobin as alkaline haematin, the standard may be employed to standardize bloods for checking other colorimetric methods of haemoglobinimetry.

SUMMARY

1. Errors may arise in the use of haemin as a standard substance in haemoglobinometry, (*a*) because the colour intensity of various samples of haemin dissolved in 0·1 N-NaOH is not strictly proportional to their iron content, (*b*) because the absorption spectrum of haemin solutions differs significantly from that of blood converted to alkaline haematin.

2. To meet this difficulty, an artificial standard has been prepared from inorganic salts which is stable, reproducible, and follows the absorption spectrum of blood in 0·1 N-NaOH between the yellow and blue-green regions.

3. Methods of application of the standard to routine haemoglobinometry are described.

4. The haemoglobin equivalent of the standard is $15{\cdot}6 \pm 0{\cdot}2$ g. of haemoglobin/100 ml. of blood, based on oxygen capacity measurements, and $16{\cdot}0 \pm 0{\cdot}2$ based on Fe determinations.

5. Determinations of Fe in ten consecutive samples of male blood gave consistently higher results (average 3%) than the values expected from oxygen capacity measurements. This difference was not due to the presence of carboxyhaemoglobin or methaemoglobin, and appears to indicate the presence of 'inactive' iron in normal male blood.

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The Chemical Estimation of Vitamin E in Vegetable Oils

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The chemical methods for estimation of vitamin E, recommended in the literature, are all based on the oxidation of a hydroquinone nucleus which is present in all modifications of tocopherol. This nucleus can either be oxidized in such a way as to give a coloured product, or its reducing power may be measured and hence the quantity of tocopherol calculated. The latter principle is employed in the gold chloride potentiometric method of Karrer & Keller (1938, 1939) and in the colorimetric methods based on the $FeCl_3$-α:α′-dipyridyl reaction of Emmerie & Engel (1938).

The method of Furter & Meyer (1939) is not specific; the red colour which develops on treatment of tocopherol with concentrated HNO_3 is given also by its biologically inactive oxidation product (Lester Smith & Bailey, 1939). A method of assaying α-tocopherol acetate by hydrolyzing with sulphuric acid and titrating with ceric sulphate, recommended by the British Pharmaceutical Codex (1942), opens new possibilities, but apparently has not yet been applied to the estimations of tocopherol in its natural sources. The gold chloride potentiometric method requires elaborate apparatus, and is more complicated than the α:α′-dipyridyl method, which is simple, rapid and accurate if interfering substances are removed from the unsaponifiable matter before reagents are applied.

In this paper we describe means for preparing suitable extracts to which the α:α′-dipyridyl reaction can be successfully applied so as to secure results consistent with biological values for natural oils as obtained by other workers (Olcott, 1934; Drummond, Singer & MacWalter, 1935) and with such tests as we have so far performed. Preliminary accounts of the work have already been given (Moore & Tošić, 1943; Tošić & Moore, 1944).

EXPERIMENTAL

Experimental basis of chemical method for estimation of vitamin E

The ability to produce a red colour with ferric chloride and α:α′-dipyridyl is not a specific property of tocopherols, but is shown by many reducing agents. Emmerie & Engel (1938) found that both carotene and vitamin A produced colour, so that it was necessary either to remove them before the estimation of vitamin E, or to make an appropriate correction. It is now clear that several other complications must be faced before it can be safely assumed that only vitamin E is being estimated. If the reaction is applied directly to oils without saponification our first consideration must be that esterified tocopherols do not produce colour. Since tocopherol is believed to be present in the free state in all its natural sources this criticism may not be serious except when we are dealing with oils artificially fortified with synthetic esters. Another objection is that oils may contain pigments which interfere with the assessment of the correction for carotenoids. These pigments, together with sterols, have been removed by Parker & McFarlane (1940) by means of extraction with 85 % H_2SO_4. We are, however, still faced with the finding of Kaunitz (1944) that the presence of oil seriously interferes with the development of colour by tocopherol. The objection to saponification, by ordinary methods, is that substantial losses of tocopherol may occur. Even in examining the unsaponifiable fraction, moreover, precautions have still to be taken. It may be necessary not only to correct for the presence of carotenoids, but in our experience to remove other reducing substances which may affect the accuracy of the method much more seriously.

For a successful application of the α:α′-dipyridyl reaction therefore it is necessary: (*a*) to saponify the oil under conditions which prevent the destruction of vitamin E, (*b*) to separate interfering substances from the unsaponifiable matter, (*c*) to make a correction for the reducing power of any carotenoids which cannot conveniently be removed, and (*d*) to protect the solution against sunlight during the estimation (Lester Smith & Bailey, 1939).

Choice of light filters

With the use of Zeiss Pulfrich Photometer, maximum transmission of the red ferrous-α:α′-dipyridyl complex formed by reaction with α-tocopherol was found to be at about 520 mμ. The most suitable filters for the colorimetric estimation should therefore be the green S 50 or S 53 filters. With an S 53 filter, a calibration curve for the reducing power of synthetic α-tocopherol proved to be a straight line for 0–168 μg. of α-tocopherol in the reagent mixture. The amount of tocopherol, as μg./ml. of solution, could be calculated by multiplying the extinction

coefficient as read on the instrument by a factor of 166·6.

In calculating the quantities of α-tocopherol, and other substances in unsaponifiable matter which reduce $FeCl_3$ in α:α′-dipyridyl reaction, this factor was always used. All the data regarding $FeCl_3$-reducing substances have been expressed in terms of 'apparent' synthetic α-tocopherol.

Stability of α-tocopherol during saponification in absence and presence of pyrogallol

Since Lester Smith & Bailey (1939) were unable to prevent the loss of tocopherol on saponification by working as far as possible in an atmosphere of inert gas, some other form of protection was necessary. A novel procedure involving the addition of pyrogallol was tried. It was expected that, during treatment of the oils with hot ethanolic potash, this substance would compete with tocopherol for the available oxygen, and so reduce the loss of vitamin. A series of recovery tests on synthetic α-tocopherol and its acetate after a saponification procedure was carried out in absence and presence of pyrogallol.

(*a*) *Recovery of α-tocopherol.* To 1 ml. of an ethanol solution of synthetic α-tocopherol, containing a quantity likely to be found in oil samples to be examined, 1 ml. freshly prepared 10% pyrogallol solution in ethanol was added. For each sample containing pyrogallol a control without pyrogallol (but with 1 ml. ethanol added) was prepared. The contents were heated on a water-bath, conc. KOH solution sufficient to saponify 1 g. of oil was added, and heating continued for a short time until saponification was complete. Distilled water was then added, and the vitamin extracted with diethyl ether. Excess of pyrogallol was readily removed by washing with water. The extracts were then evaporated under reduced pressure in a hot water-bath and the 'unsaponifiable' matter, in this case α-tocopherol only, was examined colorimetrically with $FeCl_3$-α:α′-dipyridyl reagent. The results of these tests indicate (Table 1*a*) that pyrogallol eliminates the serious losses which occur during saponification.

(*b*) *Recovery of α-tocopherol from its acetate.* When α-tocopherol acetate is saponified as described under (*a*), the α-tocopherol formed can be estimated colorimetrically in the 'unsaponifiable' matter. Losses due to saponification in the absence of pyrogallol are considerable. On the other hand, the pyrogallol procedure secures a satisfactory recovery (Table 1*b*).

The tests described under (*a*) and (*b*) show that the losses of α-tocopherol occurring during the alkaline treatment amounted on the average to about half of the vitamin added, while in the presence of pyrogallol recovery was almost complete.

Pyrogallol procedure applied to oils

The pyrogallol procedure was applied to natural oils to assess further the accuracy of the method.

Table 1. *Recovery of synthetic α-tocopherol in presence and absence of pyrogallol*

α-Tocopherol (mg. added)	Without pyrogallol: mg. found	Without pyrogallol: % recovery	With pyrogallol: mg. found	With pyrogallol: % recovery
(*a*) Recovery of α-tocopherol in absence of oil				
1·08	0·63	58·7	1·07	99·2
1·08	0·62	57·2	1·06	98·2
1·08	0·36*	33·3*	1·06	98·2
(*b*) Recovery of α-tocopherol from its acetate in absence of oil				
1·78	0·66	37·7	1·76	98·8
1·78	1·04	58·4	1·77	99·4
1·08	0·57	52·8	1·07	99·2
1·08	0·92	85·2	1·06	98·2
(*c*) Recovery of α-tocopherol from its acetate in presence of oil				
2·06†	1·84	89·3	2·02	98·2
2·06†	1·80	87·4	2·03	98·6
2·06†	1·72	83·2	2·01	97·5
2·06†	1·77	86·0	2·03	98·6

* In this case 1 ml. of distilled water was added to the ethanol solution of the vitamin.

† These figures represent total $FeCl_3$-reducing substances of the oil which are made up as follows: 1·82 mg. α-tocopherol, added in the form of its acetate, +0·24 mg. found in the arachis oil itself by our pyrogallol method.

When α-tocopherol acetate was dissolved in arachis oil, and the colorimetric estimation done on the unsaponifiable matter, the results (Table 1*c*) indicate that during saponification pyrogallol quantitatively protects both tocopherol and the other substances which reduce $FeCl_3$. During the saponification in the absence of pyrogallol the losses amounted to 10·7–16·8% (average 13·5%). These losses were much smaller than those sustained when α-tocopherol or its acetate without oil were saponified in the absence of pyrogallol (Tables 1*a* and 1*b*). Probably the oil contains substances which partially protect the vitamin during the saponification.

Table 2. *Agreement of triplicates by the pyrogallol method*

Oil	$FeCl_3$-reducing substances* (μg./g. of oil): 1	2	3	Mean	Maximum spread as % of mean
Wheat-germ (freshly extracted)	3180	3080	3100	3120	3·20
Wheat-germ (old sample)†	558	549	541	549	3·10
Tung	2391	2361	2359	2370	1·36
Soya-bean	1355	1357	1361	1356	0·44
Cotton-seed (crude)	936	969	937	947	3·48
Cotton-seed	576	594	591	588	2·56

* Correction for carotenoid pigments has been deducted from the data in this table.

† A sample of wheat-germ oil, extracted over 10 years ago and kept in a store at room temperature.

The reproducibility of results in saponification with pyrogallol was tested on several vegetable oils (Table 2). A maximum difference between $FeCl_3$-reducing substances in triplicate estimations amounted to a maximum of 3·48 %, which is within the experimental error of estimation.

For comparing saponification procedures, with and without pyrogallol, we have examined a number of freshly extracted oils (Table 3). An average loss of $FeCl_3$-reducing substances, amounting to 18·8 %, was incurred by the saponification procedure in which pyrogallol was omitted. It is of interest to notice the agreement with the data of Lester Smith & Bailey (1939), who obtained only an 87 % recovery of α-tocopherol after saponification of wheat-germ oil in which synthetic α-tocopherol was dissolved.

Table 3. *$FeCl_3$-reducing substances of freshly extracted oils*

Oil*	$FeCl_3$-reducing substances† (μg./g. of oil) With pyrogallol	$FeCl_3$-reducing substances† (μg./g. of oil) Without pyrogallol	% difference between the two methods
Wheat-germ	3120	2470	20·8
Soya-bean	1356	1070	21·0
Firsts castor	909	814	10·4
Crude linseed	729	624	14·4
Cotton-seed	696	535	23·0
Crude rape	661	512	22·5
Crude sesame	527	430	18·3
Ground-nut	268	249	7·0
Crude cocoa-butter	220	148	32·7
Coconut	nil	nil	nil
Palm-kernel	nil	nil	nil

* Examined within 1 month of extraction.

† Corrections for carotenoid pigments have been deducted; the values of these corrections are given in Table 4.

Correction for carotenoid pigments

Although the quantity of carotenoid pigments is in many cases negligible, a few oils, such as wheat-germ, soya-bean, crude linseed and rape oils contain sufficient quantities of the pigments to necessitate a correction in the total $FeCl_3$-reducing substances. For the determination of a factor which would transform the reducing activity of μg. of carotenoid pigments into μg. of α-tocopherol the following experiment was performed:

β-Carotene in ethanol in amounts ranging from 0–112 μg. was allowed to react with $FeCl_3$-α:α′-dipyridyl reagent under conditions identical with those employed in the estimation of vitamin E and the intensity of the red colour developed was determined colorimetrically. The readings were taken about 20 min. after mixing. The curve relating the concentration of carotene to the extinction coefficient was linear. 1 μg. of carotene was found to have reducing power equal to 2·5 μg. of α-tocopherol.

In our chromatographic procedure, to be described later, we found that carotene, which is always a small proportion of total carotenoids, passes readily through the column with vitamin E. Xanthophylls, however, remain adsorbed in the column, but can be eluted together with other $FeCl_3$-reducing substances. It is often desirable to estimate the reducing substances other than α-tocopherol and carotene. It is then necessary to make a correction for xanthophylls. For this purpose, having no specimen of pure xanthophyll available, we compromised by using the same correction factor of 2·5 as for carotene. The agreement of data in recovery tests before and after chromatographic separation (see later in Tables 8, 9 and 11) suggests that the error involved in this procedure is not large.

The correction for carotene is significant in the case of very few natural oils. Correction for xanthophylls found in the 'adsorbed' fraction is large, but it need be applied only in cases in which recovery of total $FeCl_3$-reducing substances is required. In several freshly extracted natural oils more than traces of carotenoid pigments, as estimated by yellow colour, were present in the unsaponifiable matter. The values of the corrections in the few samples which contained the pigments are given in Table 4. Higher values for total carotenoid pigments were obtained if the oils were saponified in presence of pyrogallol than in its absence. This, therefore, suggests that a small proportion of carotenoid pigments may be destroyed during alkaline saponification and that this can be avoided by using pyrogallol.

Table 4. *Correction for carotenoid pigments of some oils*

Oil*	Carotenoid pigments correction (μg./g. of oil) With pyrogallol	Carotenoid pigments correction (μg./g. of oil) Without pyrogallol
Wheat-germ	289	271
Soya-bean	15	13
Crude linseed	30	27
Crude rape	37	35
Others	Traces or no carotenoids were detected	

* Freshly extracted oils given in Table 3.

Loss of $FeCl_3$-reducing substances on storage of oils

The values of total $FeCl_3$-reducing substances of some oil samples decreased with the time of storage of the oil. From Table 5, it will be seen that during storage the oils lost a portion of their $FeCl_3$-reducing substances, including carotenoid pigments. The loss was not uniform with all oils, and may depend on the type of oil and the storage conditions. A loss of 17·5 % was incurred in a sample of freshly extracted wheat-germ oil, even when this was stored at a low temperature (2–4°) for a period of only 2 months. It is evident, therefore, that in recording the data for vitamin E it is necessary to give the history of the samples examined.

Table 5. *Loss of* $FeCl_3$*-reducing substances on storage of oils*

Oil	Time of examination	$FeCl_3$-reducing substances (μg./g.)	Correction for carotenoid pigments (μg./g.)
Wheat-germ	1 day after extraction	3120	289
	After storage of 60 days at 2–4°	2576	181
Wheat-germ II	Over 10 years old	549	30
	17 months later*	491	29
Tung	Over 5 years old	2370	4
	15 months later*	2039	4
Soya-bean	Within 1 month of extraction	1356	15
	15 months later*	1222	11
Crude linseed	Within 1 month of extraction	729	30
	15 months later*	694	26
Cotton-seed	Within 1 month of extraction	696	0
	15 months later*	400	0
Crude rape	Within 1 month of extraction	661	37
	15 months later*	440	24
Crude sesame	Within 1 month of extraction	527	0
	15 months later*	420	0

* Stored at room temperature.

The biological inactivity of $FeCl_3$*-reducing substances of tung oil*

Tung oil was second only to wheat-germ oil in its content of substances reducing $FeCl_3$ (Tables 2 and 3). Since this oil contained only a small amount of carotenoid pigments (correction being 4 μg./1 g. of oil) and no vitamin A, it was necessary to decide whether the rest of 2370 μg. of the $FeCl_3$-reducing substances/g. of oil was either vitamin E or 'interfering substances', if it should prove inactive in biological experiments. The unsaponifiable matter of the oil, dissolved in arachis oil, was therefore tested biologically by the method of Evans & Burr (1927).

The data of these experiments (Table 6) show that total $FeCl_3$-reducing substances of tung oil have very little biological activity. Roughly 80–90 % of the $FeCl_3$-reducing substances are inactive and should be considered as interfering substances in the chemical estimation.

For example, at a dosage of 4·48 mg./animal, it was to be expected, if all the $FeCl_3$-reducing substances were α-tocopherol, that most of the rats used would have litters. Instead, out of six animals, four showed resorption, only one gave a live litter, whilst one gave a dead litter. In another experiment, ten animals were used and they were dosed with 8·12 mg. each. It was thought that the dosage of 8·12 mg. of $FeCl_3$-reducing substances should give a 100 % response in biological experiments, even if $FeCl_3$-reducing substances are present in tung oil in the form of the less biologically active derivatives of tocopherol (β- and γ-tocopherol). But the experiments showed that out of ten animals dosed at this level only one gave a live litter, while in the rest (nine animals) resorption resulted.

Chromatographic separation of biologically inactive $FeCl_3$*-reducing substances of tung oil*

The existence of the interfering substances in the unsaponifiable matter of tung oil led to experiments with various chromatographic systems to find suitable conditions for the free passage of α-tocopherol with the removal by adsorption of 'interfering' substances.

'Floridin XS earth'-benzene system. With this system we confirmed the findings of Emmerie & Engel (1939*a*) that synthetic α-tocopherol freely passes through the column with benzene as solvent and eluent. Under such conditions 98·7 % of $FeCl_3$-reducing substances of unsaponifiable matter of tung oil unfortunately behaved in the same way. The 'Floridin XS earth'-benzene system cannot therefore

Table 6. *Biological test of unsaponifiable matter of tung oil*

Oil	Date of exp.	Dose of $FeCl_3$-reducing substances (mg./animal)	No. of animals	Resorption	Dead litters	Live litters
Tung in arachis	June 1943	4·48	6	4	1 (1)*	1 (1)*
Arachis (control)	,,	0·08	5	5	0	0
Tung in arachis	August 1943	8·12	10	9	0	1 (4)*
Arachis (control)	,,	0·08	3	3	0	0

* Figures in brackets denote the number of animals in each litter.

be used for the separation of α-tocopherol from the biologically inactive 'interfering substances' in tung oil.

Aluminium oxide-light petroleum + ethanol system. In our experience aluminium oxide, if left exposed to air, adsorbs moisture, and its adsorption activity is reduced. For the purpose of eliminating this trouble, we have used several brands of standard activated aluminium oxide which exhibited constant adsorption properties. Further, it was found that with any standard Al_2O_3 the adsorption activity was reduced with an increased concentration of ethanol added to light petroleum, which served as solvent and eluent. The degree of adsorption of synthetic α-tocopherol, as well as of other $FeCl_3$-reducing substances on aluminium oxide, will therefore depend *inter alia* on both these factors.

For more detailed study of this system the chromatographic procedure was standardized. The alumina was activated by heating in a hot-air oven at 100° for 2–3 hr., just before preparing the column, which was 50 mm. long by 10 mm. diameter. As solvent light petroleum A.R. (b.p. 40–60°) was used, with the addition of 0·0–1·5 % ethanol, depending on the experiment. 10 ml. of solvent were used for preparing the column, 5 ml. for dissolving α-tocopherol or the $FeCl_3$-reducing substances, and 15 ml. for subsequent washing of the column. The solvent was always added before the top of the column became dry. The filtration rate was 70–90 drops/min.

The latter procedure was successful when activated aluminium oxide (British Drug Houses Ltd.) was used in combination with light petroleum and 1·0 % ethanol. After the preliminary experiments the adsorption activity of this alumina was studied in relation to the concentration of ethanol added to light petroleum. Table 7*a* shows that α-tocopherol was quantitatively adsorbed by the column if the concentration of ethanol in the solvent ranges between 0–0·40 %. With ethanol concentrations ranging between 0·60–0·90 % there was partial recovery, and with 1·0 % or over a quantitative recovery. With 1 % ethanol the standard column gave quantitative recoveries with 173–1734 μg. of α-tocopherol.

When the $FeCl_3$-reducing substances of the unsaponifiable matter of tung oil are passed through this column, and the solvent used contained 1 % of ethanol, 21·3 % of these are found in the unadsorbed fraction. The adsorbed substances could be eluted with 10 ml. of light petroleum containing 20 % ethanol, and amounted to 75·7 % of the total $FeCl_3$-reducing substances found in control samples before chromatographic analysis. The total recovery after chromatographic separation therefore amounted to 97·0 %.

The necessity of selecting the correct brand of aluminium oxide is emphasized by our failure with two other preparations. A specimen made by Brockman's method adsorbed too strongly, and failed to allow the quantitative passage of α-tocopherol even at concentrations of ethanol which allow the passage of the 'interfering substances' of tung oil. A specimen from A. Wander Ltd. adsorbed weakly (Table 7*b*), and allowed a passage through the column of about 70 % of the 'interfering substances', at 0·8 % concentration of ethanol.

Table 7. *Adsorption and recovery of α-tocopherol*

Aluminium oxide-light petroleum-ethanol system

Percentage of ethanol in solvent mixture	α-Tocopherol: Added (μg.)	α-Tocopherol: Found after passing through the column (μg.)	α-Tocopherol: Found after passing through the column: Percentage of amount added
(*a*) 'B.D.H.' aluminium oxide for chromatographic analysis			
0·00	867	0	0
0·40	867	0	0
0·60	867	127	14·7
0·70	867	533	61·6
0·80	867	617	70·5
0·90	867	834	96·3
1·00	1734	1725	99·6
1·00	867	867	100·0
1·00	867	859	99·2
1·00	173	172	99·5
1·20	867	867	100·0
(*b*) Aluminium oxide (A. Wander Ltd.)			
0·00	867	0	0
0·30	867	64	7·4
0·45	867	513	59·2
0·60	867	681	78·5
0·70	867	784	90·4
0·80	867	867	100·0
0·90	867	874	100·8
1·00	867	862	99·3

Influence of α-tocopherol on the adsorption of $FeCl_3$-reducing substances of some oils, and the influence of the latter on the passage of α-tocopherol

Although the technique described in the preceding section seemed promising it remained to be decided (*a*) whether the column can separate the interfering substances of oils other than tung oil in presence and absence of synthetic α-tocopherol, and (*b*) whether the relative concentration of α-tocopherol to that of other reducing substances has any effect.

To test the influence of synthetic α-tocopherol on the behaviour of the mixed reducing substances of plant oils, wheat-germ oil, crude cotton-seed oil, and tung oil were chosen as three widely different types which contain large quantities of reducing substances. Mixtures were prepared containing about the same quantities of α-tocopherol and of the reducing substances present in the unsaponifiable matter of each oil. Each mixture was then passed through a column. The 'unadsorbed' fraction and an 'adsorbed' fraction, which was eluted with 10 ml. of light petroleum containing 20 % ethanol, were collected separately. For each test, control experiments were done with separate constituents of each system without mixing.

α-Tocopherol and the unsaponifiable matter of oils were adsorbed to the same extent whether passed through the column separately or as mixtures (Table 8). The 'adsorbed' and 'unadsorbed' fractions, moreover, showed little change in their

Table 8. *Influence of synthetic α-tocopherol on the passage, through alumina columns, of $FeCl_3$-reducing substances of unsaponifiable matter of some oils*

Sample		Total $FeCl_3$-reducing substances*: Before passing through the column (μg.)	After passing through the column: In 'unadsorbed' fraction μg.	In 'unadsorbed' fraction Percentage of total	In 'adsorbed' fraction μg.	In 'adsorbed' fraction Percentage of total	Recovery μg.	Recovery Percentage of total
1*a*	Synthetic α-tocopherol alone	434	431	99·4	0	0	431	99·4
1*b*	Unsaponifiable matter of tung oil alone	475	100	21·0	360	75·8	460	96·8
	Total	909	531	58·5	360	39·6	891	98·1
I.	Mixture of 1*a* and 1*b*	909	538	59·2	372	41·0	910	100·2
	Difference		+7	+0·7	+12	+1·4	+19	+2·1
2*a*	Synthetic α-tocopherol alone	476	470	98·8	0	0	470	98·8
2*b*	Unsaponifiable matter of crude cotton-seed oil alone	518	242	46·7	274	52·9	516	99·6
	Total	994	712	71·6	274	27·5	986	99·2
II.	Mixture of 2*a* and 2*b*	994	727	73·1	273	27·4	1000	100·5
	Difference		+15	+1·5	−1	−0·1	+14	+1·3
3*a*	Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
3*b*	Unsaponifiable matter of wheat-germ oil alone	514	330	64·3	194	37·7	524	102·0
	Total	948	762	80·4	194	20·4	956	100·8
III.	Mixture of 3*a* and 3*b*	948	736	77·7	196	20·7	932	98·4
	Difference		−26	−2·7	+2	+0·3	−24	−2·4

* Correction for carotenoid pigments deducted.

Table 9. *Recovery of synthetic α-tocopherol in presence of unsaponifiable substances of wheat-germ oil*

Sample		Total $FeCl_3$-reducing substances*: Before passing through the column (μg.)	After passing through the column: In 'unadsorbed' fraction μg.	In 'unadsorbed' fraction Percentage of total	In 'adsorbed' fraction μg.	In 'adsorbed' fraction Percentage of total	Recovery μg.	Recovery Percentage of total
1*a*	Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
1*b*	Unsaponifiable fraction of wheat-germ oil alone	1028	628	61·1	401	39·0	1029	100·1
	Total	1462	1060	72·6	401	27·4	1461	100·0
I.	Mixture of 1*a* and 1*b*	1462	1034	70·8	395	27·0	1429	97·8
	Difference		−26	−1·8	−6	−0·4	−32	−2·2
2*a*	Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
2*b*	Unsaponifiable fraction of wheat-germ oil alone	514	330	64·3	194	37·7	524	102·0
	Total	948	762	80·4	194	20·4	956	100·8
II.	Mixture of 2*a* and 2*b*	948	736	77·7	196	20·7	932	98·4
	Difference		−26	−2·7	+2	+0·3	−24	−2·4
3*a*	Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
3*b*	Unsaponifiable fraction of wheat-germ oil alone	257	151	58·9	95	37·0	246	95·9
	Total	691	583	84·3	95	13·7	678	98·1
III.	Mixture of 3*a* and 3*b*	691	579	83·8	117	16·9	696	100·7
	Difference		−4	−0·5	+22	+3·2	+18	+2·6

* Correction for carotenoid pigments deducted.

adsorptive properties when passed through the column for the second time. The ratio of α-tocopherol to other reducing substances was also without effect on their chromatographic separation (Table 9). With our standardized column consistent results were obtained with amounts of total $FeCl_3$-reducing substances from 257 to 1462 μg.

Biological activity of the two fractions of wheat-germ oil

The unsaponifiable matter of wheat-germ oil (Table 8) contained some 40% of reducing substances which were adsorbed on the column, and hence did not appear to be α-tocopherol. To confirm this conclusion biological tests were carried out upon the adsorbed and unadsorbed fractions.

Freshly extracted wheat-germ oil was saponified in presence of pyrogallol and the unsaponifiable matter was dissolved in sufficient light petroleum + 1·0% ethanol, so that 5 ml. of this solution contained about 1 mg. of $FeCl_3$-reducing substances. 5 ml. portions of this solution were chromatographically separated, each portion with a freshly made column. The unadsorbed fractions were combined, the solvent evaporated under reduced pressure and the unsaponifiable matter obtained in this way was dissolved in a suitable volume of arachis oil. Each adsorbed fraction was eluted from the column with 10 ml. portions of light petroleum containing 20% ethanol, and the eluates combined and evaporated under reduced pressure. The unadsorbed fraction obtained in this way was also dissolved in arachis oil. Before dosing, the reducing substances of the two preparations were estimated chemically.

The two fractions were tested biologically, as described earlier, against arachis oil as a control (Table 10). Treatment with the adsorbed fraction, at the dosage of 3·5 mg. of reducing substances after correction for carotenoid pigments, gave no response in any of the five rats which were used. Resorption resulted in all cases. On the other hand, animals which were dosed with 4 mg. of $FeCl_3$-reducing substances from the unadsorbed fraction gave litters, with the exception of two animals in which resorption occurred. The controls, dosed with arachis oil only, gave no response, as resorption occurred in all of five animals.

Our experiments, therefore, support the conclusion that reducing substances which appear in the unadsorbed fraction of wheat-germ oil are mainly tocopherols. Reducing substances which remain adsorbed in the column are either not tocopherol or are modifications of tocopherol which have only slight biological activity. This view is consistent with our previous finding that the reducing substances of tung oil which are mainly adsorbed on the column, show only slight activity in biological tests.

Table 10. *Biological test of 'adsorbed' and 'unadsorbed' fractions of unsaponifiable matter of wheat-germ oil*

Sample	Date of exp.	Dose of $FeCl_3$-reducing substances (mg./animal)	No. of animals	Resorption	Dead litters	Live litters
Adsorbed fraction dissolved in arachis oil	May 1944	3·50	5	5	0	0
Unadsorbed fraction dissolved in arachis oil	„	4·00	7	2 or 3	1 or 2	3 (7) (7) (2)*
Arachis oil (control)	„	0·18	5	5	0	0

* Figures in brackets denote the number of animals in each litter.

Method for the chemical estimation of vitamin E

Reagents

Pyrogallol solution. 5% (w/v) freshly prepared, with ethanol as solvent.

KOH *solution* (for saponification). Aqueous solution containing 4 g. of KOH + 2·7 ml. H_2O.

α-Tocopherol standard (for calibration purposes). Ethanol solution containing about 1 mg. of synthetic α-tocopherol/1 ml.

$FeCl_3$ *solution.* Freshly prepared 0·2% (w/v) ethanol solution.

α:α′-Dipyridyl solution. Freshly prepared 0·5% (w/v) ethanol solution. This reagent, and the $FeCl_3$, may be stored in the dark for over 2 weeks.

Ethyl ether. A.R. for extraction purposes.

Ethanol. A.R.

Light petroleum. A.R. (b.p. 40–60°).

Aluminium oxide (British Drug Houses Ltd., for chromatographic analysis). Activated by heating at 100° in an air oven for 2–3 hr. before use. It is essential to standardize each new batch of alumina against known materials, such as synthetic α-tocopherol and the unsaponifiable matter of tung oil.

Light petroleum + ethanol solvent. For chromatography, 1% (v/v) of absolute ethanol in petroleum ether; for eluting the 'adsorbed fraction' 20% (v/v) ethanol in petroleum ether.

Procedure

Saponification. 1 g. of oil is weighed in a conical flask, 2 ml. of 5% pyrogallol are added, and the mixture is heated to 86° on a water-bath. After the addition of 0·44 ml. of the KOH solution, the flask is shaken in the bath for about 2 min., which usually causes complete saponification. To the mixture 8 ml. distilled water are added, the unsaponifiable matter is extracted in a separating funnel by shaking vigorously first with 10 ml. of ether and then with 5 ml. of ether. The two ether extracts are combined and washed

twice, each time with 5 ml. of 0·5 % aqueous KOH, vigorous shaking being avoided at this stage in view of the danger of emulsions. The ether extract is next washed several times with 10 ml. portions of distilled water, with only gentle shaking for the first and second times. Adequate washing of the extract is indicated by the quick separation of layers after vigorous shaking. The ether extract is evaporated on a water-bath, under reduced pressure, and the unsaponifiable matter dissolved in a volume of ethanol, so that 1 or 2 ml. of such solution are equivalent to about 1 mg. of $FeCl_3$-reducing substances. The solution at this stage may be kept overnight in a refrigerator without harm.

If duplicate estimations are to be made, unsaponifiable matter equivalent to 2400–4000 μg. of reducing substances should be prepared. It is usually necessary to take more than 1 g. of oil, to increase proportionately the amounts of all the reagents used, and sometimes to extend slightly the time of saponification.

Chromatographic analysis. A portion of the solution of unsaponifiable matter, containing about 1 mg. of the reducing substances, is evaporated under reduced pressure to dryness on a hot water-bath, and care is taken to remove all ethanol. (*a*) *Solvent.* 30 ml. of light petroleum + 1 % ethanol solvent are prepared carefully for each chromatographic separation. (*b*) *Preparation of aluminium oxide column.* Activated aluminium oxide is packed in a tube of 10 mm. diameter and about 120 mm. long, so that length of the column is 50 mm. and diameter 10 mm. 10 ml. of light petroleum ether + 1 % ethanol solvent are passed through the column, which is now ready for use. For each estimation a new column should be made.

A portion of the unsaponifiable matter is dissolved in 5 ml. of the solvent mixture and passed through the column which is then washed with 15 ml. of the solvent. Throughout the analysis the top of the column should not be allowed to become dry, and the rate of filtration should be 70–90 drops/min. The 'unadsorbed fraction' of 20 ml. volume is collected in a clean flask, the solvent evaporated under reduced pressure on a hot water-bath, and the residue dissolved in ethanol and made up to a volume of not more than 25 ml. This solution is now ready for colorimetric estimations.

Colorimetric estimations. (*a*) *Estimation of carotenoid pigments.* In the unadsorbed fraction mainly carotene is found, and is estimated by its natural yellow colour by means of a Pulfrich photometer with the S 47 filter. The quantity of carotene is expressed in μg./1 g. of oil. (*b*) *Estimation of vitamin E.* A portion of the solution, containing between 100 and 160 μg. of $FeCl_3$-reducing substances, is pipetted out into a 25 ml. measuring flask. This is found out in a preliminary test, if the vitamin E content of the material examined is quite unknown. Sufficient ethanol is added to bring the volume to about 20 ml., then 1 ml. of $FeCl_3$ solution followed by 1 ml. of α:α'-dipyridyl solution. The volume is then made up to 25 ml. with ethanol, the contents being mixed by shaking the flasks after each addition. The control is prepared in the same way, but the unsaponifiable matter is omitted. The flasks are left in a dark place for 15 min. for full development of the colour. The unknown and the control solutions are transferred to 5 cm. long micro-cells, and their colours matched in a Pulfrich photometer with the use of the green S 53 filter. Since the solutions are sensitive to light the measurements must be made in diffused daylight or low artificial illumination.

Calculation of results. As an example, results with a sample of soya-bean oil may be quoted. 4·14 g. of oil were saponified, and the unsaponifiable matter dissolved in 4·14 ml. of ethanol. 1 ml. portions of this solution were chromatographed. The unadsorbed fraction containing vitamin E was eventually brought up to the volume of 20 ml. and carotene and vitamin E content of oil estimated.

Uncorrected vitamin E value. 6 ml. of this solution gave an extinction reading of 0·47, so that $FeCl_3$-reducing substances of this fraction amount to $0{\cdot}47 \times 166{\cdot}6 \times \frac{20}{6} = 261\,\mu g./1$ g. oil. The factor 166·6 converts the step photometer reading into μg. of α-tocopherol (see p. 499).

Carotene correction. It was found that 1 g. of oil contained 1·17 μg. of carotene; thus the correction for this pigment amounts to 2·9 μg./1 g. of oil (see p. 500).

Vitamin E. After the carotene correction is deducted, the vitamin E content of the oil comes to 258 μg./1 g. of 'apparent' α-tocopherol.

Estimation of the reducing substances of the 'adsorbed' fraction and of total $FeCl_3$-reducing substances. Although these substances are discarded in the estimation of vitamin E, their evaluation is recommended for two reasons. First, if an estimation is also made of the total reducing substances of the unsaponifiable fraction before chromatographing, a useful check is available on the recovery of reducing substances, and on the behaviour of the adsorption column. Secondly, a high proportion of adsorbed reducing substances may suggest the desirability of biological tests for tocopherol isomers other than the α-form. These isomers, which may be more strongly adsorbed, have low biological activity.

The adsorbed substances are eluted from the adsorption column with 10 ml. of light petroleum containing 20 % ethanol, and estimations are made of the yellow colour and of $FeCl_3$-reducing substances in a similar way to that described for carotene and vitamin E.

Vitamin E content of oils

The contents of vitamin E and of reducing substances other than vitamin E of numerous vegetable oils are recorded in Table 11. Wheat-germ oil, in which vitamin E made up 60 % of the total reducing substances, was the richest source. Some other oils, e.g. tung oil, soya-bean oil, contained relatively large quantities of reducing substances, but only about 20 % of these were in the form of vitamin E. An old specimen of crude cotton-seed oil, a recognized source of vitamin E, contained about one-third of the total reducing substances of fresh wheat-germ oil, and 45 % appeared to be α-tocopherol. Coconut oil and palm-kernel oil contained no reducing substances.

Tocopherol isomers

The presence in natural fats of the β- and γ-isomers of tocopherol, and possibly also of other substances having slight vitamin E activity, raises a problem which we have been unable to investigate fully, mainly on account of difficulties in obtaining the isomers in this country. Through the kindness of Prof. A. R. Todd, however, we obtained a small

Table 11. *Vitamin E and other* $FeCl_3$*-reducing substances of plant oils*

Oil	Time of examination after extraction (months)	$FeCl_3$-reducing substances: Before passing through the column (μg./g. of oil)	After chromatographic separation: In 'unadsorbed' fraction*: μg./g. of oil	In 'unadsorbed' fraction*: Percentage of total	In 'adsorbed' fraction: μg./g. of oil	In 'adsorbed' fraction: Percentage of total	Recovery: μg./g. of oil	Recovery: Percentage of total
Wheat-germ I	Within 2	2776†	**1690†**	60·9	1061†	38·2	2751†	99·1
Wheat-germ II	Over 144	491†	**286†**	58·3	160†	32·6	491†	92·0
Tung	Over 84	2039†	**444†**	21·8	1540	75·6	1984†	97·4
Soya-bean	15	1222†	**250†**	20·5	967†	79·3	1217†	99·8
Cotton-seed (crude)	Over 84	936†	**425†**	45·4	497†	53·1	922	98·5
Cotton-seed (refined)	15	400	**144**	36·0	254	63·5	398	99·5
First castor	15	693	**184**	26·5	493	71·2	677	97·7
Linseed (crude)	15	694†	**110†**	15·8	578†	83·3	688†	99·1
Maize	Over 84	458	**274**	59·8	168	36·7	442	96·5
Rape (crude)	15	440†	**110†**	25·0	331†	75·2	441†	100·2
Sesame	15	420	**40**	9·5	359	85·5	399	95·0
Arachis I	15	336	**125**	37·2	212	63·1	337	100·3
Arachis II	15	240	**108**	45·0	128	53·3	236	98·3
Cocoa-butter (crude)	15	212	**47**	22·2	156	73·6	203	95·8
Arachis III‡	15	123	**39**	31·7	81	65·8	120	97·5
Coconut	15	0	**0**	—	0	—	—	—
Palm-kernel	15	0	**0**	—	0	—	—	—

* Vitamin E fraction.

† Correction for carotenoid pigments has been deducted from the figures marked with asterisk; other oils contained either traces or no carotenoid pigments.

‡ A sample of refined undeodorized ground-nut oil for special white margarine.

specimen of the *p*-nitrophenyl-urethane of β-tocopherol. After saponification the free alcohol was run on to the adsorption column in the usual way. In contrast to the behaviour of the α-isomer only some 30 % passed through.

Further investigation into the separation of the tocopherol isomers is obviously desirable. It is fortunate, however, that the inefficient passage of β-tocopherol through the column, if confirmed, tends to compensate for its much lower biological activity, which according to Mason (1944) is only 40 % of that of α-tocopherol. As far as the α- and β-isomers are concerned, therefore, our routine method should give a good indication of the total biological activity, as usually expressed in terms of α-tocopherol.

SUMMARY

1. A method based on the α:α′-dipyridyl-$FeCl_3$ reaction has been evolved for the estimation of vitamin E in vegetable oils.

2. To avoid oxidation of the vitamin and other $FeCl_3$-reducing substances, the oils have been saponified in the presence of pyrogallol.

3. In addition to the known interference by carotenoid pigments, it has been found that the unsaponifiable fractions always contain varying amounts of other irrelevant substances which are capable of reducing $FeCl_3$. These are presumably of low or negligible biological activity, and have been removed by adsorption on a column of activated alumina, under conditions which were carefully adjusted to allow the free passage of α-tocopherol.

4. Values for vitamin E, 'total reducing substances' and the interfering substances have been estimated for many oils. Tung oil gave a high value for total reducing substances, but only 20 % appeared to be in the 'α-tocopherol fraction', and the biological activity of the unsaponifiable fraction as a whole was correspondingly poor. In wheat-germ oil the 'α-tocopherol fraction' represented some 60 % of the total reducing substances. Biological tests were consistent with the concentration of activity in this fraction, and its absence from the remaining 40 % of reducing substances, which were separated by the adsorption column.

5. Further investigation into the separation of tocopherol isomers is obviously desirable. In our method β-tocopherol is partially adsorbed on the column. In practice, however, this inefficient passage through the column tends to compensate for its much lower biological activity.

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The Free Amino Groups of Insulin

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That the free amino groups in proteins may be the ϵ-amino group of lysine was first suggested by Skraup & Kaas (1906), who failed to isolate lysine from deaminized proteins. Van Slyke & Birchard (1913) later found an apparent equality between the free amino-N of proteins and one-half of their total lysine-N, which suggested that the free amino groups were due exclusively to such a source. Methods were then developed to confirm the presence of the free ϵ-amino group of lysine in proteins; these consisted in treating a protein with a reagent that would react with free amino groups to give derivatives that were stable to acid hydrolysis, so that on hydrolysis of the substituted protein the derivative of lysine could be isolated. The most satisfactory was that of Gurin & Clarke (1934), who treated gelatin with benzenesulphonyl chloride, hydrolyzed the resulting benzenesulphonyl gelatin and isolated ϵ-benzenesulphonyllysine from the hydrolysate.

In some proteins, and particularly in insulin, the number of free amino groups (Van Slyke) is far in excess of that which can now be ascribed to lysine, which suggests that the protein must contain residues of certain amino-acids which are condensed in such a way that their α-amino groups remain free. Jensen & Evans (1935) have, in fact, been able to isolate the phenylhydantoin of phenylalanine from a hydrolysate of insulin that had been treated with phenyl*iso*cyanate, thus demonstrating that some free amino groups of insulin are present on phenylalanyl residues. Chibnall (1942) has suggested that the free amino groups of proteins over and above the ϵ-amino groups of lysine are due to terminal residues of polypeptide chains, and that the number of these groups must therefore give a measure of the number of polypeptide chains in the protein.

We found that methanesulphonyl amino-acids were stable to acid hydrolysis and that they could be fractionated by partition chromatography (Gordon, Martin & Synge, 1943) with an indicator in the aqueous phase of the column. The rates at which the bands moved on the columns were similar to those of the acetamino-acids. The method was satisfactory when applied to synthetic peptides, but with insulin the number of terminal groups estimated by titration of the methanesulphonyl amino-acids was far less than that suggested by the method of Van Slyke. The procedure was accordingly abandoned.

Abderhalden & Stix (1923) attempted to use 2:4-dinitrochlorobenzene (DNCB) for the identification of the terminal groups of a partial hydrolysate of silk fibroin. They did not meet with much success, chiefly owing to the presence of anhydrides in the hydrolysate and the difficulties of separating the products. It seemed, nevertheless, worth while to investigate this reagent, especially as all the 2:4-dinitrophenyl-amino-acids (referred to henceforth as DNP-amino-acids) produced are bright yellow, thereby facilitating chromatographic separation. DNCB will not react with amino-acids in $NaHCO_3$ solution unless heat is applied, and this brings about a certain amount of hydrolysis of the protein. Fortunately, however, the corresponding fluoro-compound, 2:4-dinitrofluorobenzene (DNFB) was found to react readily at room temperature, and the use of this has met with considerable success, for the DNP-amino-acids produced can be estimated

colorimetrically and separated almost completely from one another by partition chromatography. The solvent systems normally used for separating the acetyl-derivatives were not entirely satisfactory for the DNP-monoamino-acids, and several new systems had to be introduced; nevertheless, the method finally adopted embraced all amino-acids, though this was not possible with the methanesulphonyl derivatives.

The method as applied to insulin consisted of three stages. In the first the protein was treated with DNFB, hydrolyzed and the resulting coloured compounds separated chromatographically. The identification of these was based on band rates, and was confirmed by mixed chromatograms. Secondly, knowing which DNP-derivatives were present, one could assess the amount of each with reasonable accuracy by separating the fraction quantitatively and estimating the material present colorimetrically, using the pure DNP-amino-acid as a standard. Thirdly, the whole operation was carried out on a larger scale, so that the DNP-amino-acids could be isolated and satisfactorily characterized. The procedure should be applicable to the identification and estimation of the terminal residues in all peptides and proteins.

EXPERIMENTAL

Preparation of DNP-amino-acids

In order to work out a comprehensive chromatographic method for the identification of any DNP-amino-acid by its behaviour on a partition column, it was necessary to prepare the derivatives of all the naturally occurring amino-acids. Not all of these were obtained in pure form or completely characterized, as this was unnecessary for a study of their chromatographic behaviour; nevertheless, all the derivatives corresponding to those obtained from treated insulin were carefully purified, so that they could be used as standards of reference.

The derivatives of glycine, *dl*-alanine, *dl*-valine, *dl*-leucine and *l*-asparagine were prepared by the method of Abderhalden & Blumberg (1910). By a similar method, the derivatives of *dl*-methionine, *l*-proline, *l*-tryptophan, *dl*-aspartic acid, *l*-cystine and *l*-lysine were prepared but not completely characterized. The methods of purification and characterization of these compounds will be published later.

N-2:4-*Dinitrophenyl*-l-*phenylalanine*. 0·2 g. *l*-Phenylalanine and 0·4 g. $NaHCO_3$ were dissolved in 5 ml. water and to this was added a solution of 0·4 g. (0·28 ml.) DNFB in 10 ml. ethanol. The mixture was shaken for 2 hr. at room temperature, concentrated to remove ethanol, dissolved in water and extracted with ether to remove excess DNFB. The aqueous solution was then acidified, which caused the separation of an oil that immediately solidified in an amorphous form. The N-2:4-*dinitrophenyl*-l-*phenylalanine* was recrystallized twice from aqueous methanol. Yield, 0·27 g.; m.p. 186°. (Found: C, 54·2; H, 4·2; N, 12·6%. $C_{15}H_{13}O_6N_3$ requires C, 54·4; H, 3·9; N, 12·7%.)

The derivatives of *dl*-serine, *dl*-threonine, *l*-hydroxyproline, *l*-glutamic acid and *l*-arginine were prepared in a similar way using DNFB. Their purification and characterization will be published later. The acid equiv. wt. (277) of the serine derivative showed that it was mono-DNP-serine (271), and that DNFB does not react with aliphatic hydroxyl groups.

ε-N-2:4-*Dinitrophenyl*-l-*lysine*. Several attempts to prepare this compound by the action of DNCB or DNFB on the copper complex of lysine proved unsuccessful. It was eventually prepared from the α-acetyl-compound as follows. 0·48 g. α-acetyl-*l*-lysine (Neuberger & Sanger, 1943) and 0·75 g. $NaHCO_3$ were dissolved in 3 ml. water. To this was added a solution of 0·5 g. DNCB in 10 ml. ethanol. The mixture was then heated under reflux on a water-bath for 4 hr. The ethanol was removed by evaporation *in vacuo* and the residue dissolved in water, filtered to remove excess DNCB and acidified with HCl while hot. An oil separated, which was extracted into chloroform. The chloroform solution, after drying with anhyd. Na_2SO_4, was taken to dryness, leaving an oil. On rubbing this with dry ether the α-*acetyl*-ε-*DNP*-l-*lysine* crystallized and could be recrystallized from a mixture of ethanol and ether: m.p. 110°; yield 0·7 g. (75% theoretical). 0·2 g. of the compound was boiled under reflux for 3 hr. with 5 ml. 20% HCl. On cooling, the ε-*DNP*-l-*lysine hydrochloride* separated as crystals containing 1 mol. of water of crystallization. After standing overnight it was filtered off and washed with very little water and ethanol and then thoroughly with ether. It was recrystallized from dilute HCl; m.p. 186°. (Found: C, 39·0; H, 5·5; N, 15·6; Cl, 9·5%. $C_{12}H_{16}O_6N_4.HCl.H_2O$ requires C, 39·5; H, 5·2; N, 15·4; Cl, 9·8%.)

α-N-2:4-*Dinitrophenyl*-l-*lysine*. It is generally believed that the lysine residues in proteins are combined through the α-amino groups while the ε-amino groups are free. One would thus expect to isolate only ε-DNP-lysine from a hydrolysate of a DNP-protein. It is not impossible, however, that some, at least, of the lysine residues in proteins are combined through the ε-amino group, in which case α-*DNP-lysine* would be formed on hydrolysis of a DNP-protein. This second derivative was accordingly synthesized as follows. 1·25 g. ε-benzoyl-*l*-lysine were treated for 4 hr. with 1·0 g. DNCB as described above. On acidification an oil separated which solidified on standing. It weighed 2·0 g. 0·5 g. of this α-*DNP*-ε-*benzoyl*-l-*lysine* was boiled under reflux for 3 days with a mixture of 5 ml. acetic acid and 5 ml. conc. HCl. The mixture was then evaporated to dryness, taken up in water and filtered to remove unchanged substance. On neutralization with pyridine the α-DNP-*l*-lysine crystallized out in 60% yield; m.p. 260° (decomp.). (Found: C, 45·6; H, 5·3; N, 17·5%. $C_{12}H_{16}O_6N_4$ requires C, 46·2; H, 5·1; N, 17·9%.)

Tyrosine derivatives. When *l*-tyrosine was treated with DNCB in excess two coloured compounds were produced, which moved down a 1% butanol-chloroform column at $R=0·5$ and 0·2 respectively. With DNFB only the faster-moving compound was produced. The acid equiv. wt. (519) indicated that this was *O*:*N*-diDNP-*l*-tyrosine (513), which had been prepared by Abderhalden & Stix (1923). The slower-moving compound was presumably *N*-DNP-*l*-tyrosine, since O-*DNP*-l-*tyrosine* is colourless. The monoDNP-tyrosine prepared by Abderhalden & Stix (1923) was presumably the *O*-DNP-derivative in an impure form.

O-2:4-*Dinitrophenyl*-l-*tyrosine*. Since DNFB reacts with the hydroxyl group of tyrosine, a hydrolysate of a DNP-protein would be expected to yield *O*-DNP-*l*-tyrosine. It

was accordingly necessary to synthesize this latter compound. 0·55 g. *N*-acetyl-*l*-tyrosine (du Vigneaud & Meyer, 1932) was treated for 4 hr. with 2·0 g. DNCB as described above. The solution was only faintly coloured at the end of the reaction, in contrast to the bright colours obtained when DNCB reacts with amino groups. On acidification an oil was produced which partly crystallized. From aqueous ethanol the *N-acetyl-O-DNP-l-tyrosine* crystallized in white needles; m.p. 194°; yield 0·1 g. The product was boiled for 3 hr. under reflux with 20% HCl; on cooling and evaporating to a small volume, a precipitate was obtained which was filtered off, dissolved in warm dilute HNO_3 and neutralized with pyridine while hot. *O*-DNP-*l*-tyrosine crystallized in white needles containing 1 mol. of water of crystallization; m.p. 202° (decomp.). (Found: C, 49·9; H, 4·2; N, 11·9%. $C_{15}H_{13}O_7N_3.H_2O$ requires C, 49·4; H, 4·1; N, 11·6%.)

Reaction of DNFB with histidine. By the action of DNCB on histidine Abderhalden & Blumberg (1910) obtained two compounds containing one and two DNP-groups respectively. I find that on treatment with excess DNFB only the diDNP-histidine is obtained, indicating that DNFB reacts with the imidazole ring. As this is free in proteins it seemed probable that a monoDNP-histidine would be formed on hydrolysis of a DNP-protein. In an attempt to prepare this compound, α-acetylhistidine (Bergmann & Zervas, 1928) was treated with DNFB, but there was very little reaction and only a small amount of a yellow oil was obtained. A portion of this was hydrolyzed with 20% HCl and the products studied chromatographically. Two compounds were obtained. The first of these was not extracted from acid solution into ether and moved fast on a 17% butanol-chloroform column; it was not observed when the hydrolysis products of DNP-insulin were treated. The second was ether-soluble and moved fast on a chloroform column. It seemed probable that this latter might be 2:4-dinitroaniline, derived by breakdown of the substituted histidine, and it was indeed found to have the same *R* values on ethanol-ligroin and acetone-cyclohexane columns as the aniline derivative itself. This same band was obtained on hydrolysis of both diDNP-histidine and DNP-insulin. These results indicate that DNFB reacts only slowly with α-acetylhistidine, in contrast to the almost quantitative reaction with histidine itself. It is interesting to note that benzoylation of the imidazole group of histidine is affected by the presence of other polar groups in the molecule (Gerngross, 1919) and that the same appears to be true on reaction with DNFB. To ascertain how histidine would react if present in a polypeptide chain with its carboxyl as well as its amino group in peptide linkage, the action of DNFB on α-benzoylhistidine methyl ester (Gerngross, 1919) was studied; the reaction was very slow and the same products were given on hydrolysis as with α-acetylhistidine. Finally, to ascertain how histidine would react if present as a terminal residue in a peptide chain with only the amino group free, the reaction with histidine methyl ester (Pauly, 1904) was also studied. In this case the product obtained on hydrolysis consisted entirely of the diDNP-derivative, indicating easy reaction with the imidazole group. To summarize, it may be inferred that when histidine is present in the body of a polypeptide chain of a protein or as a terminal residue with its carboxyl group free, the imidazole group will react slowly with DNFB so that on hydrolysis some 2:4-dinitroaniline will be obtained together with a possible unknown breakdown product which is insoluble in ether and moves fast on a 17% butanol-chloroform column. If histidine is present as a terminal residue with its amino group free the diDNP-derivative will be obtained.

Reaction of DNFB with —SH *groups.* Saunders (1934) showed that DNCB reacts more readily with —SH groups than with amino groups, so that one is led to infer that when a protein containing —SH groups is treated with DNFB and hydrolyzed, *S*-DNP-cysteine would be produced. Saunders (1934) prepared a derivative by the action of DNCB on cysteine, which he stated crystallized with difficulty. I have repeated the preparation and find that the product does not give a positive ninhydrin reaction and is extracted from dilute HCl into ether. On a 3% butanol-chloroform column it forms two bands having $R = 1{\cdot}2$ and 0·3 respectively. It would appear, therefore, that the condensation does not run smoothly, and the product does not exhibit the expected properties.

To obtain information as to the compound to be expected from DNP-proteins, a small amount of the *S*-DNP-derivative of reduced glutathione was prepared and hydrolyzed for 6 hr. with 20% HCl. *S*-DNP-glutathione is a well-characterized product of known structure (Saunders, 1934). The hydrolysate appeared to contain one substance which moved fast down a 66% methyl ethyl ketone (M.E.K.)-ether column and at $R = 0{\cdot}08$ in 17% butanol-chloroform. It was not extracted from dilute HCl into ether. It is suggested that this band represents *S*-DNP-cysteine, and that the substances prepared by the action of DNCB on cysteine are formed from *S*-DNP-cysteine by the action of alkali. More experimentation is necessary to verify this, but it appears that the product obtained from glutathione would also be obtained from a protein.

Chromatographic separation of DNP-derivatives

In general the partition chromatographic method of Gordon *et al.* (1943) was employed to separate the DNP-derivatives, using a stationary aqueous phase adsorbed on the silica gel and a moving organic phase. It was, of course, unnecessary to use an indicator in the aqueous phase as the derivatives

themselves are coloured. To describe such solvent systems the terminology of the above-mentioned workers is followed, i.e. 1% butanol-chloroform refers to a solution in chloroform of butanol, 1% (by vol.) subsequently saturated with water. In some cases glycol or an aqueous organic solvent such as ethanol or acetone was employed as the stationary phase, and a non-polar solvent in equilibrium with it as the moving phase. Such systems do no more than indicate the relative rates of the different derivatives on the columns, and R values alone cannot be used for identification unless a parallel experiment with a sample of the authentic derivative itself is run.

The data given in Table 1 show that all the amino-acid derivatives listed can be separated by one or other of the solvent systems employed, the sole exception being leucine and *iso*leucine. The method

Table 1. *Band rates of ether-soluble DNP-amino-acids*

(Figures are values of R—Gordon *et al.* 1943.)

Stationary phase ...	Water		Water	Water	Water	Ethanol	Methanol	Acetone	Glycol
Mobile phase ...	Butanol-chloroform		Chloroform	5% propanol-cyclohexane	33% ether-ligroin	Ligroin	Carbon tetrachloride	Cyclohexane	Benzene
	3%	1%							
2:4-Dinitroaniline	—	—	Fast	—	—	0·35	—	0·8	—
DNP-leucine	—	Fast	0·45	Fast	Fast	0·75	0·6	0·5	0·65
DNP-*iso*leucine	—	Fast	0·45	Fast	Fast	0·8	0·6	0·55	0·6
DNP-valine	—	Fast	0·4	Fast	Fast	0·55	0·5	0·4	—
DNP-phenylalanine	—	Fast	0·2	Fast	Fast	0·5	0·4	0·25	—
DNP-methionine	—	Fast	0·25	Fast	1·0	0·3	0·4	—	—
DNP-proline	Fast	0·7	0·2	1·0	0·9	0·2	0·3	0·4	0·3
DNP-alanine	Fast	0·7	0·15	1·0	1·0	0·25	0·25	0·4	0·2
DNP-tryptophan	—	—	0·06	0·9	0·8	0·1	—	—	—
DNP-glycine	—	0·5	0·08	0·6	0·6	0·1	—	—	0·07
diDNP-lysine	—	0·5	0·06	Insol.	0·25	Insol.	—	—	0·3
diDNP-tyrosine	—	0·5	0·08	Insol.	0·25	—	—	—	0·5
DNP-hydroxyproline	—	0·13	0·02	—	—	—	—	—	—
DNP-threonine	—	0·07	—	0·2	0·2	—	—	—	—
DNP-serine	—	0·05	—	Insol.	0·1	—	—	—	—
DNP-glutamic acid	0·4	0·03	—	0·35	0·3	—	—	—	—
DNP-aspartic acid	0·4	0·02	—	0·2	0·2	—	—	—	—
diDNP-cystine	0·35	—	—	Insol.	0·0	—	—	—	—

are especially useful for the separation of the mono-amino-acid derivatives. To prepare an ethanol-ligroin column, 1 vol. water, 1 vol. ethanol and 10 vol. ligroin (b.p. 80–100°) are shaken together until the two phases are in equilibrium. The lower aqueous phase is used as the stationary phase of the column, 1 ml. being added to each 2 g. dry silica, and the upper ethanol-ligroin phase is used as the moving phase of the column. A methanol-carbon tetrachloride column is prepared as above from 1 vol. water, 1 vol. methanol and 15 vol. carbon tetrachloride; and an acetone-cyclohexane column from 1 vol. water, 1 vol. acetone and 10 vol. cyclohexane. To prepare a glycol-benzene column, glycol and benzene are shaken together; 1 ml. of the glycol layer is added to each 1 g. dry silica, and the benzene layer used as the moving phase.

In Table 1 are shown the R values for the various ether-soluble DNP-amino-acids using various solvent systems. These values have been found to vary considerably with such factors as the particular batch of silica or solvent and with the distance travelled down the column. It is probable that there is a certain amount of adsorption of the compounds on the silica. The figures in Table 1, therefore, may thus be capable of estimating the component amino-acids given on hydrolysis of a protein, but the more quantitative aspects of such a procedure have not yet been fully explored. All the derivatives shown in Table 1 are extracted from acid solution into ether, and move fast on an ether column. This fact is made use of to separate them from the acid-soluble derivatives, the R values for which on various solvents are given in Table 2.

Table 2. *Band rates of acid-soluble DNP-amino-acids*

(Figures are values of R—Gordon *et al.* 1943.)

	Developing solvents		
	Methyl ethyl ketone-ether	Butanol-chloroform	
DNP-amino-acids	66%	17%	30%
diDNP-histidine	Fast	Fast	—
S-DNP-cysteine	Fast	0·08	Fast
α-DNP-arginine	0·35	—	0·2
ε-DNP-lysine	0·20	—	0·15
α-DNP-lysine	0·11	—	—

Attempts to separate the DNP-derivatives by adsorption chromatography have not been very successful. When magnesium oxide or alumina were used as adsorbents decomposition took place. It was found possible, nevertheless, to separate the glycine and leucine derivatives in acid solution on a column of talc and in ether solution on a column of calcium carbonate, in neither case, however, so effectively as in the above-mentioned partition chromatography. Likewise the use of partition chromatography using filter paper (Consden, Gordon & Martin, 1944) or starch (Synge, 1944) was not very satisfactory, due to 'tailing' of the spots or bands.

Estimation of DNP-amino-acids

The DNP-amino-acids, dissolved in N-HCl (about 0·1–0·3 mg. amino-acid-N/100 ml. N-HCl), were estimated colorimetrically with a photoelectric absorptiometer. In the case of DNP-phenylalanine the solubility in this solvent was too low, and 1% $NaHCO_3$ had to be used. Standard curves were plotted for the derivatives of the naturally occurring amino-acids. These varied considerably from one to another, and differences were noted between the curves representing the *l* and *dl* samples of the same amino-acid. In all cases the colour intensities do not obey Beer's law.

Stability of DNP-derivatives to acid hydrolysis

Since the liberation of DNP-amino-acids from a DNP-protein requires acid hydrolysis, it was necessary to investigate the stability of the former products during such treatment. To effect this, a standard solution of the derivative was made up

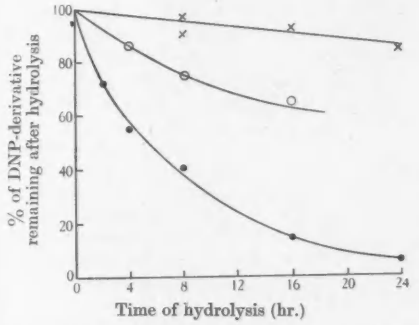

Fig. 1. Rate of hydrolysis of DNP-amino-acids with boiling 20% (w/v) HCl. • DNP-glycine; ⊙ DNP-phenylalanine; × ε-DNP-lysine.

and samples were hydrolyzed with 20% HCl for varying periods. These were then taken to dryness, purified on a suitable column and the amount of unchanged derivative estimated. The results of such experiments with DNP-phenylalanine, DNP-glycine and ε-DNP-lysine are shown in Fig. 1.

Approximate experiments with the DNP-derivatives of alanine, valine, arginine, serine, tryptophan, aspartic acid and diDNP-lysine indicated less than 20% breakdown in 8 hr. It would thus appear that, with the single exception of DNP-glycine, all the derivatives exhibit satisfactory stability for the purpose intended.

Preparation of DNP-insulin

0·5 g. crystalline insulin and 0·5 g. $NaHCO_3$ were dissolved in 5 ml. water; 10 ml. ethanol and 0·5 ml. DNFB were then added and the mixture mechanically shaken for 2 hr. The DNP-insulin, which had precipitated as an insoluble yellow powder, was centrifuged down and washed with water, ethanol and ether, and air dried.

For the experiments which follow it was necessary to know the insulin content of a given sample of DNP-insulin. This could not be ascertained directly by a determination of N, as the nitro-groups would interfere, and it was therefore computed indirectly from an estimation of the amide-N. To do this a sample of the DNP-insulin was boiled for 3 hr. with 2 N-HCl and, after neutralization, the liberated NH_3 estimated in the micro-Kjeldahl apparatus using a borate buffer of pH 8·5. A control experiment with asparagine showed that treatment with DNFB did not alter the amide-N content as determined in this way. In one particular experiment, where all the values quoted below are calculated on a moisture-free basis, the sample of DNP-insulin contained 1·07% amide-N and 0·04% amino-N (Van Slyke). Since insulin itself contains 1·38% amide-N (Chibnall, 1942; Rees, unpublished), it follows that 100 mg. of the DNP-insulin correspond to 77·2 mg. of insulin. If the amino-N (Van Slyke) value for insulin be taken as 0·85% (Chibnall, 1942) it would appear that 93% of the free amino groups of the insulin have been substituted.

Identification of the terminal residues in insulin

100 mg. DNP-insulin were boiled under reflux for 8 hr. with 10 ml. 20% (w/w) HCl. After cooling it was extracted three times with ether. This separated the hydrolysate into two fractions; the ether extract, which contained the DNP-derivatives listed in Table 1, and the aqueous solution which contained free amino-acids and any of the DNP-derivatives listed in Table 2.

The ether extract was taken to dryness, and the material passed through an ether column to remove HCl, which would cause adsorption of the derivatives at the top of the subsequent column. The material was then fractionated on a chloroform column. There was a faint band that moved fast

and so could not be an amino-acid derivative; it seems probable that it was 2:4-dinitroaniline, derived from the breakdown of a substituted histidine derivative. It could not be separated from 2:4-dinitroaniline by the use of an ethanol-ligroin or an acetone-cyclohexane column. The rest of the colour was in two bands having $R = 0{\cdot}2$ and $0{\cdot}1$ respectively. Each was 'run out' of the column separately, taken to dryness and samples used for the following experiments.

Fraction 1 (R in chloroform $= 0{\cdot}2$) formed one band having $R = 0{\cdot}5$ on an ethanol-ligroin column, and one band having $R = 0{\cdot}3$ on an acetone-cyclohexane column. These rates suggested that the material was DNP-phenylalanine, which was confirmed by the following mixed chromatograms. A sample mixed with DNP-leucine formed two bands having $R = 0{\cdot}6$ and $0{\cdot}4$ respectively on an ethanol-ligroin column. Another sample mixed with DNP-alanine formed two bands on an ethanol-ligroin column having $R = 0{\cdot}4$ and $0{\cdot}2$ respectively. A third sample mixed with DNP-valine formed two bands having $R = 0{\cdot}5$ and $0{\cdot}3$ respectively on an acetone-cyclohexane column. On mixing with authentic DNP-phenylalanine only one band was obtained on each of the above columns. Since the original hydrolysis was carried out for only 8 hr. it was possible that either band could represent a DNP-derivative of a peptide. In order to show that fraction 1 was not a peptide, a sample was hydrolyzed for a further 8 hr. with 20% HCl, extracted with ether and analyzed on the various columns given above. Only one band was obtained and the R values were unchanged. If it had been a peptide one would have expected to find evidence for a new band, representing the terminal group. It is, of course, possible that fraction 1 contained a DNP-phenylalanyl-peptide that is inseparable from DNP-phenylalanine on the columns used, but this seems unlikely and does not alter the fact that DNP-phenylalanine is present.

A sample of fraction 2 (R in chloroform $= 0{\cdot}1$) on a 5% propanol-cyclohexane column gave one band having $R = 0{\cdot}6$, and on a 33% ether-ligroin column one band having $R = 0{\cdot}55$. These rates suggested that the material was DNP-glycine and this was confirmed by the following mixed chromatograms. A sample mixed with DNP-alanine gave two bands having $R = 0{\cdot}3$ and $0{\cdot}2$ respectively on a chloroform column. Mixed with diDNP-lysine, two bands having $R = 0{\cdot}6$ and $0{\cdot}2$ respectively were formed on a 33% ether-ligroin column. Mixed with DNP-tryptophan two bands were formed which moved at $R = 0{\cdot}8$ and $0{\cdot}5$ respectively in 5% propanol-cyclohexane. When mixed with DNP-glycine only one band was obtained on each of the above columns. Further hydrolysis of the material brought about no change, showing that it was not a DNP-peptide.

After thorough ether extraction the original aqueous solution was still brightly coloured, indicative of substances listed in Table 2. The presence of ϵ-DNP-lysine was to be expected, and in order to ascertain what others might be there, a sample of the acid solution was taken to dryness and its behaviour on various columns studied. On a 66% M.E.K.-ether column one band was formed, having $R = 0{\cdot}2$. In 30% butanol-chloroform it moved at $R = 0{\cdot}2$. When mixed with α-DNP-arginine, two bands having $R = 0{\cdot}35$ and $0{\cdot}2$ respectively were formed on a 66% M.E.K.-ether column. These facts indicated that ϵ-DNP-lysine was the only coloured DNP-derivative in the aqueous solution.

The above experiments showed that the hydrolysate of DNP-insulin contained DNP-phenylalanine, DNP-glycine, ϵ-DNP-lysine, and no other coloured DNP-derivative.

Estimation of the amino-acids representing terminal residues in insulin

After the identity of the DNP-amino-acids produced on hydrolysis of DNP-insulin had been established, the next step was to estimate them quantitatively. It can be seen from Fig. 1 that DNP-glycine is relatively unstable to acid hydrolysis, so that if a time period of as long as 8 hr. is used a rather large correction for breakdown must be applied. Estimations were accordingly made after shorter periods of hydrolysis. In each case it was necessary to test the various fractions for the presence of peptides as described in the previous section. No evidence for these was obtained after hydrolysis for 4 hr. but after only 2 hr. a phenylalanyl-peptide was detected in fraction 2. This was inseparable from DNP-glycine on a chloroform column, but could be separated on a special acetone-cyclohexane column made from 1 vol. water, 3 vol. acetone and 10 vol. cyclohexane. The peptide moved at $R = 0{\cdot}7$ and DNP-glycine at $R = 0{\cdot}45$. To estimate the DNP-phenylalanine contained in the peptide, it was subjected to a further 4 hr. hydrolysis.

The experimental details were as follows. Three samples of about 100 mg. DNP-insulin were boiled under reflux with 10 ml. 20% HCl for 8, 4 or 2 hr. respectively. In each case the acid solution was extracted five times with ether, the ether extracts being washed with water and the washings returned to the original aqueous solution. The collected ether extracts were reduced to dryness, passed through an ether column prepared from 2 g. silica, evaporated to dryness and fractionated on a 6 g. chloroform column. The two fractions representing DNP-phenylalanine and DNP-glycine were separately collected and taken to dryness. In the 2 hr. hydrolysate the DNP-glycine fraction was purified on an acetone-cyclohexane column (containing 3 vol. acetone). The DNP-phenylalanine

fraction was dissolved in 1% $NaHCO_3$, made up to 50 ml. and estimated colorimetrically. The DNP-glycine fraction was made up to 25 ml. with N-HCl. As standards the pure derivatives of the naturally occurring amino-acids were used.

The aqueous solution, after extraction with ether, was taken to dryness and subjected to a further 8 hr. hydrolysis with 20% HCl to break down any lysine peptides; this should not greatly affect the lysine estimation as the ε-DNP-derivative is relatively stable (Fig. 1). The acid solution was then made up to 100 ml., 10 ml. samples taken, reduced to dryness and passed through a 66% M.E.K.-ether column. The band was 'run out', taken to dryness and made up with N-HCl to 25 ml. for estimation.

Table 3. *Estimation of the free amino groups of insulin*

Time of hydrolysis (hr.)	Fraction	Amino-N as % insulin-N	Amino-N corrected for hydrolysis	No. of groups per insulin submolecule (mol. wt. 12,000)
8	Glycine	0·67	1·65	2·2
	Phenylalanine	1·00	1·35	1·8
	Lysine	1·16	1·3	1·7
4	Glycine	0·86	1·5	2·0
	Phenylalanine	1·05	1·2	1·6
	Lysine	1·32	1·4	1·9
2	Glycine	1·13	1·55	2·1
	Phenylalanine	1·07	—	—
	Phenylalanine from peptide	0·32	—	—
	Total phenylalanine	1·39	1·5	2·0
	Lysine	1·43	1·55	2·1
Mean	Glycine	—	—	2·1
	Phenylalanine	—	—	1·8
	Lysine	—	—	1·9

The results of the three experiments are set out in Table 3. The values given in column 3 represent the free amino-N of the amino-acid as a percentage of the original insulin-N. In column 4 these values are corrected by means of the data given in Fig. 1 for the breakdown which the derivatives are assumed to have undergone as a result of the hydrolysis; the corrections may not be strictly valid as the stability of the derivatives while still condensed in the protein or in peptide split-products may not be the same as that found for the derivatives themselves. In column 5 the corrected values are presented in terms of amino-acid residues per insulin submolecule of mol. wt. 12,000 (Chibnall, unpublished). It can be seen that within the limits of experimental error there are two residues of each amino-acid per insulin submolecule.

Isolation of DNP-amino-acids representing the terminal residues in insulin

To confirm the identity of the three DNP-derivatives obtained from insulin it was necessary to carry out an experiment on a larger scale and isolate the compounds concerned in a pure form. A 4 hr. period of hydrolysis was chosen for reasons given above.

1·03 g. air-dried DNP-insulin (corresponding to 0·755 g. moisture-free insulin) were boiled under reflux with 100 ml. 20% HCl for 4 hr. and the cooled hydrolysate extracted with ether as described above. The ether extract was taken to dryness and the material first passed through an ether column and then fractionated on a chloroform column prepared from 40 g. silica. The DNP-phenylalanine and DNP-glycine fractions were collected and worked up as follows.

N-2:4-*Dinitrophenyl*-l-*phenylalanine*. The chloroform solution was taken to dryness and the material passed through a 20 g. acetone-cyclohexane column to ensure purification. The coloured solution was taken to dryness and dissolved in a minimum of warm dilute $NaHCO_3$ solution. This solution was acidified and after standing for a few days crystals of DNP-phenylalanine had separated. The crude product weighed 14·0 mg. (38% of the material present as estimated colorimetrically). It was recrystallized from aqueous methanol and then from a mixture of acetone and cyclohexane, yielding 10·1 mg. of material which melted at 186° alone or when mixed with authentic DNP-phenylalanine. (Found: C, 55·2; H, 4·1%. Calc. for $C_{15}H_{13}O_6N_3$: C, 54·4; H, 3·9%.)

N-2:4-*Dinitrophenylglycine*. After evaporation of the chloroform solution, the whole fraction was purified on an acetone-cyclohexane column (containing 3 vol. acetone). The DNP-glycine fraction was taken to dryness, and crystallized from hot water; yield 10·5 mg. Another 5·3 mg. were obtained by working over the mother liquors, making a total yield of 15·8 mg. (90% of the amount estimated). Abderhalden & Blumberg (1910) report a m.p. of 205° for DNP-glycine. The purest samples I have synthesized usually decompose between 195 and 202° according to the rate of heating. To make the necessary comparison three melting-point tubes containing synthetic DNP-glycine, the above crystalline material and a mixture of the two respectively were heated side by side in the melting-point apparatus. They all decomposed simultaneously at 195°. (Found: C, 40·0; H, 2·9%. Calc. for $C_8H_7O_6N_3$: C, 39·8; H, 2·9%.)

ε-N-2:4-*Dinitrophenyl*-l-*lysine*. The aqueous solution containing the ε-DNP-lysine was taken to dryness and hydrolyzed for a further 20 hr. with 20% HCl. It was then evaporated to a small

volume and allowed to stand overnight. An amorphous yellowish grey precipitate had formed, which was filtered off. The nature of this precipitate, which weighed 100 mg., is not yet clear. The filtrate was then taken to dryness. A column was next prepared, using 40 g. silica, on which were adsorbed 20 ml. N-HCl, 66 % M.E.K.-ether being used as the mobile phase. The ε-DNP-lysine fraction was then dissolved in 2 ml. water, which were mixed with 4 g. dry silica, suspended in the solvent, poured on to the previously prepared column and developed with 66 % M.E.K.-ether. This procedure was found more satisfactory than the normal one in the presence of large amounts of free amino-acids. As it passed out of the column the yellow material was collected in three fractions. Each of these was separately evaporated to dryness and dissolved in warm dilute HCl, filtered and allowed to evaporate slowly in a desiccator. The first fraction produced well-formed crystals of the hydrochloride, the second rather impure crystals and the third fraction would not crystallize. This suggested the presence of some colourless impurity that moves on the column at a rate somewhat slower than ε-DNP-lysine; it may have been *O*-DNP-tyrosine. On working over the various fractions, and applying chromatography to those known to be somewhat impure, there were finally obtained 10·2 mg. of pure crystals and another 14 mg. of cruder crystals, making a total yield of 24 mg. (55 % of the amount estimated). The pure material melted at 186°, alone and when mixed with authentic ε-DNP-lysine hydrochloride. (Found: C, 39·6; H, 5·4 %. Calc. for $C_{12}H_{16}O_6N_4$. HCl.H_2O: C, 39·5; H, 5·2 %.)

DISCUSSION

Under the conditions used in the above-mentioned experiments DNFB reacts with amino, phenolic-hydroxyl, thiol and possibly imidazole groups. The *O*-DNP-tyrosine formed by the reaction with phenolic-hydroxyl groups is colourless and thus does not interfere with the estimation of the coloured amino-derivatives. The nature of the reaction with —SH groups is not quite clear, but it appears that *S*-DNP-cysteine should be distinguishable from other DNP-derivatives chromatographically. It was not present in the insulin experiment. The reaction with the histidine imidazole group appears to be very slow, unless histidine is present as a terminal residue with its amino group free.

The results with insulin show that in every insulin submolecule of mol. wt. 12,000, two of the free amino groups are located on glycine residues, two on phenylalanine residues and two represent the ε-amino groups of lysine residues. The presence of free amino groups of phenylalanine has already been demonstrated by Jensen & Evans (1935), though not quantitatively, but terminal glycine residues have not been previously reported, nor has it been shown before that the ε-amino groups of lysine are free. The present results indicate that there are six free amino groups per submolecule of insulin. From amino-N determinations by the Van Slyke method, Chibnall (1942) suggested the presence of 21 free amino groups per molecule of mol. wt. 36,000 or seven per submolecule of 12,000. This rather higher value can probably be accounted for by the presence of glycine as a terminal residue, since glycyl-peptides have been shown to give abnormally high amino-N values by the Van Slyke method (Abderhalden & Van Slyke, 1911; Schmidt, 1929). According to Schmidt, glycylglycine gives about 135 % of the theoretical value in 10 min. so that two terminal glycine residues would react as 2·7 amino groups. Using these enhanced values for glycyl-peptides, and those of Greenstein (1933) for lysyl-peptides, it is possible to compute the course of the reaction of insulin under the Van Slyke conditions. As shown in Table 4, the agreement between the computed and experimental values is reasonable.

Table 4. *Van Slyke amino-N of insulin*

Reaction time (min.)	Apparent no. of amino groups per submolecule of mol. wt. 12,000				Found by Van Slyke method
	2 glycyl-residues	2 phenylanalyl-residues	2 lysyl-residues	Total	
6	2·5	2·0	1·9	6·4	5·9
11	2·7	2·0	2·0	6·7	6·7
30	2·7	2·0	2·0	6·7	7·1

The presence of four free α-amino groups suggests that the submolecule is built up of four open polypeptide chains bound together by cross-linkages, presumably chiefly —S—S— linkages. It is, of course, possible that other chains may be present in the form of a ring structure with no free amino groups.

SUMMARY

1. A new method is described for the identification and estimation of the free amino groups of proteins and peptides by the formation of derivatives with 2:4-dinitrofluorobenzene.

2. The method has been applied to insulin. Assuming a minimum mol. wt. of 12,000, it is shown that six free amino groups are present; two of these are located on glycine residues, two on phenylalanine residues and two represent the ε-amino groups of lysine.

3. The results suggest that the insulin submolecule of mol. wt. 12,000 is made up of four open peptide chains, two of these having terminal glycyl-

residues and the other two terminal phenylalanyl-residues respectively.

I wish to express my thanks to Dr B. C. Saunders for kindly preparing the 2:4-dinitrofluorobenzene for me, to Prof. A. C. Chibnall for his advice and encouragement, and to the Medical Research Council for maintenance and expenses grants during the initial stages of this work.

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Growth and Diabetes in Normal Animals Treated with Pituitary (Anterior Lobe) Diabetogenic Extract

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When a dog receives daily injections of a diabetogenic extract of anterior pituitary tissue his body weight may rise during the course of the treatment despite the induction of an intensely diabetic condition (Young, 1937, 1938). This phenomenon, which is curiously in contrast with the behaviour of the dog rendered diabetic by pancreatectomy, may well be relevant to the fact (Young, 1939) that the diabetogenic agent in anterior pituitary extracts, effective in the partially depancreatized rat (Shipley & Long, 1938) or in the intact adult dog* (Young, 1939), has not, as yet, been clearly separated from the pituitary substance which induces growth and nitrogen retention in the rat—an animal which is not sensitive to the diabetogenic action of anterior pituitary extract unless the major part of the pancreas is first removed. Moreover, to the daily injection of a pituitary extract which is highly diabetogenic in the adult dog, the puppy responds with an accelerated rate of growth unaccompanied by symptoms of diabetes (Young, 1941). Daily treatment for 4 months or more, i.e. until the animal has become a dog rather than a puppy, ultimately may bring about a condition in which the diabetogenic action of the extract becomes manifest and the body weight ceases to rise, though a further increase in weight can then be brought about by simultaneous treatment with pituitary extract and insulin (Young, 1944).

Preliminary communications (Young, 1942) have briefly recorded the observation that when an adult dog, consuming a limited and constant daily amount of meat just sufficient to maintain a steady body weight under normal conditions, is given daily injections of diabetogenic pituitary extract, a rise in body weight and the appearance of symptoms of diabetes are observed. The investigations described in the present paper were undertaken in order to ascertain the nature of the metabolic changes which, in the intact dog under pituitary influence, bring about a rise of body weight and excretion of sugar even in the absence of extra food. In this connexion, investigations concerning the influence of treatment with diabetogenic pituitary extract on cats and on rats have also been carried out.

* In the present paper the term 'diabetogenic' connotes ability to induce hyperglycaemia and glycosuria (with or without ketonuria) in an intact normal animal (adult dog or cat).

METHODS

Chemical

The N content of urine, faeces, tissues and food was determined by the Kjeldahl method.

Urine. Sugar was estimated by Benedict titration and ketone bodies by the Denigès-Van Slyke method, β-hydroxybutyric acid being oxidized with hot acid dichromate solution. The results are expressed as 'total equivalent acetone'.

The D/N quotient for a diabetic animal (i.e. the number of g. of urinary glucose divided by the number of g. of corresponding urinary N) was calculated and corrected for preformed dietary carbohydrate as described by Marks & Young (1939).

Blood. Sugar was determined by a modified Hagedorn-Jensen method, and ketone by the method of Crandall (1940).

Tissues and foodstuffs. The methods for the estimation of starch (or glycogen), total carbohydrate, fat (ether-soluble material), water and ash are those of Marks & Young (1939).

Anterior pituitary extract. This was the Seitz-filtered alkaline extract of fresh ox pituitary (anterior lobe), the preparation of which has previously been described (Young, 1941). The final concentration was such that 4 ml. contained the material extracted from 1 g. of fresh tissue. It was administered by subcutaneous injection with aseptic precautions. During control periods the same amount of 0·9% NaCl or calf-thymus extract (prepared analogously with the pituitary extract) was usually administered as of the pituitary extract.

In the intact dog weighing 10–15 kg. the diabetogenically effective dose of the pituitary extract, administered repeatedly by the subcutaneous route, is 5 ml., and this was the volume injected daily unless otherwise stated. With the 3 kg. cat the corresponding dose was 1 ml., while the rat received 0·5 ml. daily unless otherwise stated.

Biological

General. In these experiments intact adult dogs and cats, and both young and adult rats, have been employed. Both sexes were used though male animals were preferred, since the sexual cycle was capable of introducing uncontrollable variations into experiments with female animals. The dogs were mainly terriers weighing 10–15 kg., while the rats were of the albino type bred originally from Wistar animals by the Medical Research Council. All animals were weighed daily and in some instances daily rectal temperatures and pulse rates were also recorded.

Blood samples were obtained from the marginal ear veins in dogs and cats, and in rats from the heart immediately after death.

Throughout each experiment the environmental conditions were controlled as rigidly as possible and when necessary the temperature of the animal rooms was controlled thermostatically.

At the conclusions of the experimental periods the animals were killed by a heavy dose of nembutal, administered intravenously or intraperitoneally, in order to obtain tissues for analytical purposes. In one experiment with rats (see Table 9), groups of the dead animals were skinned and passed several times through a mechanical mincer with frequent stirring to obtain a homogeneous mass, samples of which could be analyzed for N, fat and ash.

Food. Dogs received a diet consisting of a mixture of raw lean horse muscle and boiled ox liver. The proportions offered varied according to the animal's proclivities but were constant for each individual. A typical mixture consumed by a 10 kg. dog was 400 g. of raw muscle and 100 g. of cooked liver; this contained, on the average, 110 g. of protein, 7·5 g. of fat, and 4·9 g. of carbohydrate (expressed as glycogen), and yielded 603 kg. cal. (calc.) on biological oxidation.

Since cats and rats, unlike dogs, were inclined to select the separate constituents of their diet, they were given an intimately blended mixture. This contained, per 100 g., 73 g. of lean horse muscle, 24 g. of boiled ox liver, 2 g. of crude cod-liver oil, 0·5 g. of 'Marmite' (Marmite Food Extract Co. Ltd.) and 0·5 g. of bone meal. Its mean constitution was 21·1% protein, 3·7% fat and 0·9% carbohydrate, and it yielded 152 kg. cal./100 g. (calc.).

Each animal received a weighed amount of food every day and any residue unconsumed at the end of 24 hr. was removed and weighed. A sample of the food was always allowed to remain outside an animal cage during each 24 hr. period in order to allow assessment of the desiccation which had occurred in any unconsumed residue. In general, however, the animals consumed the whole of their rations.

Water was always given *ad libitum*, the amount taken being measured in some experiments.

Maintenance of animals. In the majority of experiments the animals were kept in metabolism cages, the urine being collected under toluene. In some experiments the faeces were also collected.

In the growth tests with rats the animals were fed on a strictly controlled amount of food, so that it was necessary to keep the individual animals in separate cages even though the results were treated as those for a group.

In a large proportion of the experiments recorded here the animals were maintained at a steady body weight, under normal conditions, by being allowed to eat daily a fixed amount of food which was sufficient, but only just sufficient, to keep the body weight constant. The requisite amount of food was determined during a preliminary period before the experiment proper started. Fully grown, adult animals were allowed, during this period, to consume food *ad libitum*, the amount eaten each day being recorded. For a period of at least a week, during which the body weight remained sensibly constant, the mean food intake was thus determined. This mean amount was then fed daily during the remainder of the experiment. With young growing rats the daily food intake during the preliminary period was restricted by stages until the body weight had attained an approximate constancy, the daily ration at this point being then fixed for the duration of the experiment.

With dogs and rats only very seldom was an animal found which would not eat a constant amount of food daily throughout the experiment and in general the animal's appetite was enhanced by the pituitary treatment unless substantial ketosis developed. With cats, on the other hand, few animals were found which would eat the same daily ration of food for some weeks. Results are recorded only for those experiments in which the food intake was constant throughout.

Determination of nitrogen balance. In the present series of experiments N balance was determined only with dogs and cats. Comparable results with rats have previously been described (Cuthbertson, Webster & Young, 1941).

After the animal had attained a steady body weight with food *ad libitum*, and was consuming a fixed and daily ration of food which was just sufficient to maintain this weight, the first control period of the experiment was begun. This period lasted 1–3 weeks and during this time the amount of urinary N eliminated each day was determined. Since

the body weight was constant, the assumption could be made that the animal was in N equilibrium, an assumption that was confirmed in a number of instances by direct observation (see below). If the animals were in nitrogen equilibrium the mean urinary N excretion during the control period could be taken to represent an N balance of zero, and any subsequent change in urinary N elimination resulting from treatment with pituitary extract could be interpreted in terms of N balance by reference to this basal value.

Several assumptions are implicit in the above argument: (1) the mean urinary N excretion during the control period was indeed representative of nitrogen equilibrium; (2) the N content of the food was constant throughout the period of the experiment; (3) faecal N was substantially constant and was not altered by treatment of the animal with pituitary extract. Verification of these assumptions is embodied in the typical data in Table 1. These show: (1) that during the control period the mean N balance, directly determined, was not significantly different from zero; (2) that the mean N content of the food consumed during the control and experimental periods was the same. (Although some variation was inevitably found from time to time in the N content of food rations, since large amounts of food were always prepared at one time and thoroughly mixed it was small and quite insignificant with respect to the magnitude of other uncontrollable biological errors); (3) that the faecal N excretion was not significantly altered by the administration of anterior pituitary extract to the animal (cf. Gaebler & Galbraith, 1941). It may be noted that in a series of control investigations on ten dogs the faecal N averaged 2·1 g./day (max. 4·7; min. 0·7) while the animals, whose mean body weight was approximately 14 kg., were receiving an adequate diet of meat. This is equivalent to the faecal loss of 94 mg. of N/g. of dietary N (max. 152; min. 57), so that even if slight fluctuations had occurred in faecal N as the result of experimental treatment of the animal the changes would not have invalidated the demonstration of a pituitary-induced N retention, which sometimes exceeded 15 g./day.

Since the assumptions considered above appeared to be valid, faecal N was not assayed in the majority of the experiments here recorded, the change in N balance induced by pituitary treatment being assessed on the basis of changes in urinary N only.

Miscellaneous. (*a*) *Calculation of calorific value of food.* The factors employed were: carbohydrate, 4 kg. cal./g.; protein, 4 kg. cal./g.; fat, 9 kg. cal./g.

(*b*) *Calculation of surface area of the body.* For dogs the formula $S = 0{\cdot}112\sqrt[3]{W^2}$, and for cats $S = 0{\cdot}099\sqrt[3]{W^2}$ (Lusk, 1928), gave the surface area (S) in sq.m., W being the body weight in kg. For rats the formulae, based on the work of Lee & Clark (1929), were $s = \frac{3{\cdot}336w^{0{\cdot}94}}{l}$, or $s = 12{\cdot}54w^{0{\cdot}60}$, where s = surface area in sq.cm., w = body weight in g. and l = body length (tip of nose to anus) in cm.

(*c*) *Measurement of dimensions and volume of the body.* Measurements of the body length, tail length and the length of specific bones were carried out by direct mensuration on skiagrams of rats after death (cf. Griffiths & Young, 1942). The volume of the body of the dead rat was measured by observation of the direct displacement of water on immersion; the mean specific gravity of the body substances thus became calculable.

(*d*) *Recording of voluntary activity of animals.* With rats this presented no difficulty since they were easily trained to run in a revolving cage carrying a revolution counter. For dogs, a metabolism cage was so pivoted as to rock slightly as the dog moved across the midline of the cage. Electrical contacts were attached so that the rocking of the cage charged and discharged a condenser, the discharge actuating an electrical counter. I am greatly indebted to Dr R. B. Bourdillon for suggesting this device and for assistance with its construction.

(*e*) *Statistical analysis of results.* The '*t*' test was employed, a difference between two mean values being regarded as significant if P was no greater than 0·05.

Table 1. *Nitrogen balance in dog* 132

(Data given are the mean values (together with the standard errors of the means where appropriate) for periods of about 1 week.)

Period	Duration of period (days)	Mean N content (g./day) of: Food	Urine	Faeces	Mean N balance (g./day): *A**	*B**
Control	8	31·8	30·8	1·5±0·29	(0·0)	−0·5±1·4
Pituitary treatment	5	31·8	22·9	1·4±0·18	8·4	7·5±2·0†
			Difference	0·1±0·34		8·0±2·4†

* A = difference between mean urinary nitrogen for control period and that for pituitary period. B = difference between food nitrogen and the sum of the urinary and faecal nitrogens.

† Unless the N excretion and retention after pituitary treatment has begun can be expected to be constant from day to day the standard error of the mean value may yield a misleadingly low estimate of the significance of the difference. Observation shows that N retention is not constant but rises to a maximum value 4–5 days after the initiation of pituitary treatment. Despite this the values of standard errors show the mean N retentions to be statistically highly significant.

RESULTS

A. Experiments with dogs

The inverse relationship between weight increase and intensity of diabetes in treated animals. When an adult dog is given a constant daily amount of meat which is just sufficient to maintain a steady body weight during a suitable control period, the nature of his response to the daily injection of diabetogenic anterior pituitary extract over a period of a week depends on a number of conditions, which include

the age and general condition of the animal. A very young adult may react like the puppy (Young, 1941, 1944) and fail to exhibit diabetes as the result of the administration of a dose of diabetogenic pituitary extract which is effective in more mature animals. Such an animal will usually, however, exhibit glycosuria as the result of prolonged and vigorous treatment, and will respond to the daily administration of a supraminimal dose over a shorter period. In pituitary extract. When a dog has received repeated injections of pituitary extract he sometimes becomes insensitive to further treatment with small doses and fails to show N retention, body-weight increase, or diabetes. Such an animal will sometimes, though not always, respond if the amount of administered pituitary extract is substantially increased, but with some animals a large enough dose to evoke such a response could not be given. The origin of this resistance is obscure, though it might be the result of anti-hormone formation (cf. Dohan & Lukens, 1939). If the

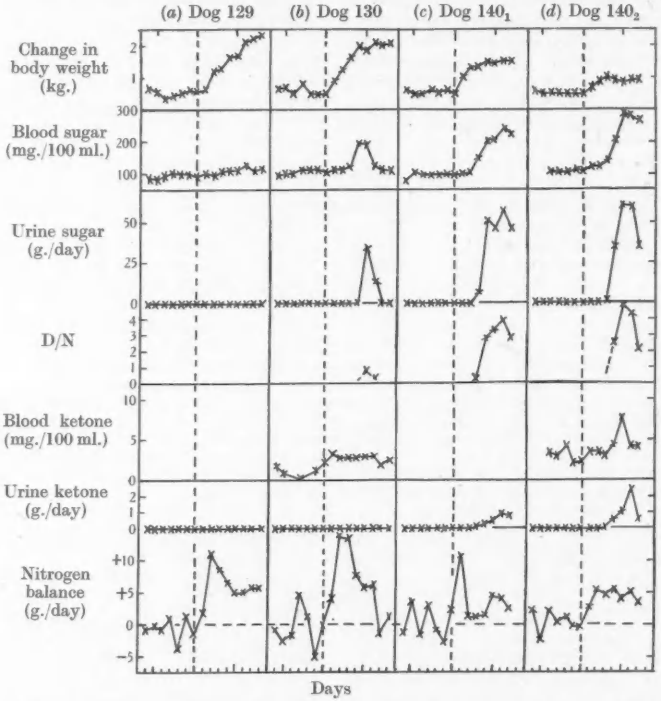

Fig. 1. Influence of daily injections of diabetogenic anterior pituitary extract on the four dogs. Each animal consumed the same daily ration of food (meat—yielding on the average approx. 1300 kg. cal./day/sq.m. of initial body surface) throughout the whole period of the experiment. The vertical dotted lines indicate the first day of the series of daily subcutaneous injections of 5 ml. of pituitary extract.

Fig. 1 is illustrated the results of four typical experiments which were carried out under similar conditions. All the dogs received a constant daily ration of food throughout, and all were in good condition. In each instance the animal responded at once to the daily injection of 5 ml. of anterior pituitary extract (equivalent to 0·25 g. of fresh ox anterior lobe) by exhibiting N retention and a rise in body weight.

The experiments illustrated in Fig. 1 may be taken as typical of the various types of reaction which are evoked when the adult dog, receiving a constant daily amount of food, is treated daily with small doses of diabetogenic animal is allowed to remain untreated for some months his sensitivity to the diabetogenic and N-retaining action of the anterior-pituitary extract returns. For reasons such as these it is important that, if a comparison is to be made between different animals (cf. Fig. 1), the dogs should all either have been previously untreated with diabetogenic extract, or have been left 'fallow' for a period long enough to enable the effects of any previous treatment to have subsided completely.

Dog 129 (Fig. 1), a young adult, responded to the daily injection of 5 ml. of extract for 1 week, by a continuous increase in body weight, the N balance remaining positive all the time. With dog 130, on

the other hand, the body weight rose sharply during the first 5 days of treatment, but thereafter did not increase significantly. On the fifth day of the administration of extract the blood-sugar level had risen to 200 mg./100 ml. and sugar was found in the urine over the subsequent 3 days. Despite continuation of the daily injections of extract the glycosuria subsided on the eighth day of treatment, at which time the positive N balance had also disappeared.

In the first experiment on dog 140 (Fig. 1*c*) a significant rise in body weight occurred over a period of only 3 days; by the fourth day the blood sugar was elevated and the glycosuria, which then appeared, persisted throughout the remainder of the experimental period. Ketonuria, which had been absent in the previously described experiments on dogs 129 and 130, appeared also on the fourth day of this experiment and thereafter persisted. Despite the glycosuria and ketonuria, N balance remained positive during the whole of the experimental period, although the body weight rose little after the third day of pituitary injections. In a second experiment with dog 140 (Fig. 1*d*) the total increment in body weight was less, though the weight showed a significant gain during the first 3 days of the period of pituitary treatment. On the sixth day of pituitary injections the blood sugar approached 300 mg./100 ml., and glycosuria had reached 60 g./day, with a corrected D/N quotient of 4·69; by the seventh day the excretion of ketone bodies exceeded 2 g./day, although the blood ketone level was not high in this experiment.

The results of these experiments indicated that the rise in body weight almost ceased when the blood sugar rose to hyperglycaemic levels with consequent glycosuria. If, as with dog 129, a diabetic condition did not supervene, the animal's body weight, like that of the puppy (Young, 1941, 1944), continued to rise throughout a limited period of pituitary treatment so that the total increment in body weight was usually greater than that of the animals which developed a diabetic condition, with consequent cessation of the rise in weight. Thus it is clear that the magnitude of the increase in body weight and the time of appearance of the diabetic condition were, in general, inversely related. The quicker the response to the diabetogenic action of the extract, the less did the body weight rise before diabetes supervened and the weight ceased to augment. It seemed desirable, therefore, to study the N retention during pituitary treatment in some detail in those animals which became diabetic as the result of daily injections of pituitary extract.

Retention of nitrogen in dogs becoming diabetic as the result of pituitary extract. Fig. 2 illustrates typical results of an experiment in which a severely diabetic condition developed in a dog given daily injections of anterior pituitary extract for a period of 1 week, the diabetes disappearing within 4 days of cessation of treatment. The daily food intake was rigidly constant throughout the period illustrated. When daily injections of 5 ml. of pituitary extract were begun the body weight rose sharply, but by the fourth day of treatment the weight had steadied at the level then obtaining and hyperglycaemia, with a resulting glycosuria, became manifest, followed later by a substantial ketonuria. Polyuria was also observed during the period of glycosuria, though the immediate response to the first injection of pituitary extract was a fall in urine volume (cf. Gaebler, 1935). From the latter observation it seemed probable that some part of the initial steep rise in body weight was the result of a temporary retention of water; the results of a study of the water balance in these animals will be reported elsewhere. Throughout the whole of the 7-day period of pituitary injections in this experiment the N balance was positive despite the induction of an intensely diabetic condition with a D/N quotient as high as 3·69. In their experiments with normal dogs under treatment with diabetogenic anterior pituitary extract, Dohan, Fish & Lukens (1941) observed that 'nitrogen retention was found during the injection period when the glycosuria attributable to protein sources was not high'. It should be emphasized that in our experiments N retention has consistently been observed as the result of the administration of pituitary extract to normal dogs on a diet of meat even though a pituitary-induced diabetes of great intensity has developed, with a D/N quotient well above that customarily regarded as maximal. There is no evidence on which an unequivocal interpretation of such a high D/N quotient can be based, since the glycogen stores of these pituitary-treated diabetic animals are large (Houssay, Biasotti & Dambrosi, 1936; Young, 1939) and any washing out of stored carbohydrate would elevate the D/N quotient. Nevertheless, it seems certain that N retention occurred at a time when protein must have been the main source of the sugar excreted by our meat-fed dogs.

Within 4 days of the cessation of pituitary treatment the glycosuria, ketonuria and polyuria had all disappeared, while the blood sugar had reverted to a normal level (Fig. 2). Since both the body weight and the N balance were subject to daily variation it is not possible to determine precisely on which date the body weight started to decline and N to be lost, but it is clear that both these processes did not begin until some days after the disappearance of glycosuria and polyuria. Two weeks after the cessation of pituitary injections the animal's body weight had almost returned to the initial, pre-experimental, level, while the N balance was still slightly negative.

It has previously been recorded that when normal dogs are given daily injections of diabetogenic pituitary extract a proportion of them exhibit a diabetic condition which is only transitory (Young, 1937, 1938). In such an animal a suitable increase in the amount of extract injected daily usually brings about the reappearance of the symptoms of diabetes (Young, 1937, 1938). Fig. 3 illustrates the results of an experiment, in which body weight, blood sugar and ketone, urinary sugar and ketone, and water and N balances, were all simultaneously determined daily for a period of nearly 9 weeks in an animal consuming a constant daily ration of food throughout the experiment. The body weight rose sharply when the daily injection of pituitary extract began, but although hyperglycaemia developed and glycosuria appeared on the fourth and fifth days of treatment (the body weight having then steadied at its new level) both had disappeared by the eighth day of treatment. The N balance became strongly positive immediately after the daily injection of pituitary extract had begun, but, despite the continued injection of the same dose of extract, N equilibrium had been re-established by the seventh or eighth day of the experiment, at which time the animal had become resistant to the diabetogenic action of the extract. Thus the response of the animal to pituitary diabetogenic influence disappeared at the same time as the animal ceased to retain N under the influence of the pituitary extract. On the eighth day of treatment the amount of extract injected daily was doubled. The response to this augmented dose was a gain in body weight, associated with a positive N balance and a slight but delayed rise of blood sugar and blood ketone. But the rise of blood sugar was insufficient to induce glycosuria, and on the sixteenth day of the experiment the daily dose of extract was again doubled. This doubling resulted in a further increase in body weight, a delayed and slight hyperglycaemia and glycosuria, and some small retention of N. Again, however, the glycosuria and hyperglycaemia disappeared despite continuation of pituitary treatment at the high level then attained, and on the

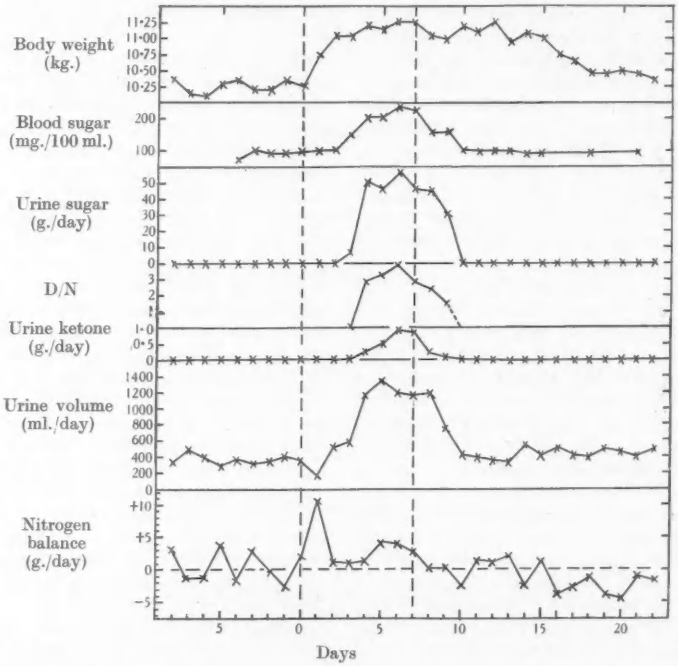

Fig. 2. Influence of daily injections of diabetogenic anterior pituitary extract, for a period of 1 week, on dog 140. Throughout the period illustrated the animal consumed the same daily ration of meat food. This yielded 1240 kg. cal./day/sq.m. of initial body surface. The first vertical dotted line (day 0) indicates the initiation of a series of daily subcutaneous injections of 5 ml. of pituitary extract, while the second vertical dotted line (day 7) indicates the day on which the injections ceased, i.e. no pituitary extract was given on day 7 and thereafter.

twenty-fourth day of the experiment the treatment with pituitary extract stopped. This resulted in a negative N balance and a fall of weight, but the body weight was still nearly 3 kg. above the initial (pre-experimental) level 9 days after cessation of pituitary treatment, and appeared to have steadied.

that there was nothing grossly abnormal in the chemical constitution of the tissues of this animal.

The results of these experiments indicated that a positive N balance was maintained throughout the period of effective treatment with a diabetogenic extract, despite the fact that in those animals which

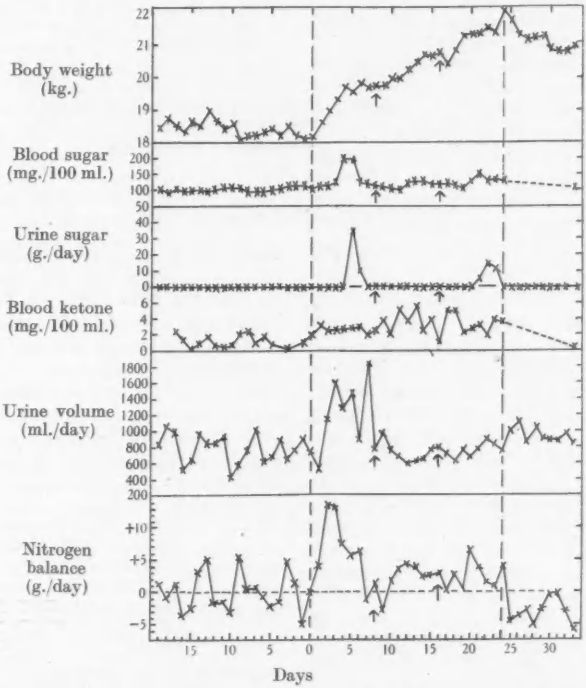

Fig. 3. Influence of daily injections of diabetogenic anterior pituitary extract on dog 130. Throughout the period illustrated the animal consumed the same daily ration of meat food. This yielded 1420 kg. cal./day/sq.m. of initial body surface. The first vertical dotted line (day 0) indicates the initiation of a series of daily injections of pituitary extract. The daily dose was originally 5 ml. but was doubled on each of the days indicated by the arrows (day 8 and day 16). Pituitary extract was not given on the day indicated by the second vertical dotted line (day 25) and thereafter.

At this time the animal was killed after starving for 16 hr. Samples of liver and muscle tissues were analyzed, with the following results:

	Content (%) of			
	Glycogen	N	Fat	Water
Liver	9·62	2·83	2·64	67·3
Muscle	0·84	3·44	1·76	72·9

The liver was enlarged in that it constituted 4·35 % of the body weight (normal, 2·62 ± 0·12 %—Table 2), and its glycogen content was high. Otherwise the only apparent significant deviations from the normal in this animal (cf. control data in Table 2) were the liver-protein and muscle-fat contents, both of which were low. It was clear, however, that there was nothing grossly abnormal in the chemical constitution of the tissues of this animal.

The results of these experiments indicated that a positive N balance was maintained throughout the period of effective treatment with a diabetogenic extract, despite the fact that in those animals which became diabetic the body weight almost completely ceased to rise after development of diabetes. The fate of the N thus retained was not at all clear, and it seemed desirable to determine the composition of the tissues of dogs whose body weights had been increased as the result of daily treatment with pituitary extract over a period of constant food intake at the pre-injection level.

Composition of the tissues of pituitary-treated dogs. Six dogs, treated daily with pituitary extract during a period of constant food intake, were killed after over-night starvation, and their tissues analyzed. The mean results for the group are compared in Table 2 with those for a control group of five similar dogs untreated with pituitary extract, standard

Table 2. *Composition of liver and muscle tissues in normal dogs and in dogs receiving daily injections of anterior-pituitary extract*

(The data given are the mean results (together with the standard errors of the means where significant differences exist) for groups of five control and six pituitary-treated dogs. The pituitary-treated animals received a constant daily amount of food throughout the experiment averaging 1354 kg. cal./day/sq.m. of body surface. Their mean initial body weight was 15·09 kg., and as the result of daily pituitary injections over a period of 30 days it rose by 2·27 kg. (15 %).)

Tissue	Group	Weight of organ (% of body weight)	Content (%) of				Total accounted for (%)	Calculated energy content (kg. cal./100 g.)
			Glycogen	N	Fat	Water		
Liver	Control	2·62±0·12	5·39	3·16±0·09	1·98±0·25	68·7	95·8	118
	Pituitary	4·72±0·39	5·75	2·86±0·09	3·16±0·35	70·1	96·9	123
	Difference	2·10±0·41	—	0·30±0·13	1·18±0·43	—	—	—
Muscle	Control	—	0·84	3·37	2·45	73·2	97·5	110
	Pituitary	—	0·91	3·26	2·39	74·4	98·1	107

(Only significant differences are indicated.)

errors of the mean results being given where the means for two groups are significantly different. It will be seen that a significant effect of the pituitary treatment is to be found only in the liver, which contains a greater percentage of fat and a smaller percentage of protein in the treated dogs. Furthermore, the livers of the treated animals are significantly enlarged (cf. Lee & Freeman, 1940). There is an apparent slight, but not statistically significant, rise in the water content of the liver and muscle tissues of the pituitary-treated dogs (Table 2). Although the percentage content of protein in the liver is diminished as the result of pituitary treatment the total amount of hepatic protein present in the animal is raised, since the liver has increased in size more than the protein content has diminished (see below). It is clear, therefore, that there is a rise in the amount of true tissue in the animal as the result of pituitary treatment, and that the increase in body weight is not merely the result of an unphysiological process such as waterlogging of the tissues. That true tissue had been added was in keeping with the non-oedematous rotundity of the pituitary-treated dogs as compared with the control animals. The former resembled over-fed pets despite their strictly limited food intake.

Correlation between nitrogen retention and increase in tissue in pituitary-treated dogs. From the data in Table 2 we can calculate the amount of N which must be retained in order that 100 g. of tissue (liver plus muscle) may be added under the influence of pituitary treatment at a time when the food intake is constant.

Thus each 100 g. of dog has become 115 g. as the result of pituitary treatment (Table 2) and, of this 115 g., 4·72 % (or 5·43 g.) is liver substance. The added liver tissue amounts therefore to $5{\cdot}43 - 2{\cdot}62 = 2{\cdot}81$ g./100 g. of original dog (i.e. dog before pituitary treatment), and the N added with this amount of liver $= \frac{(5{\cdot}43 \times 2{\cdot}86) - (2{\cdot}62 \times 3{\cdot}16)}{100} = 0{\cdot}0725$ g. If we assume that all the remainder of the 15 g. increment in body weight has the composition of muscle tissue, the N content being the average value for muscle in the two groups given in Table 1, then this addition of tissue entails

Table 3. *Relationship between nitrogen retained and body weight gained by dogs under the influence of anterior-pituitary treatment*

(Data given are the mean values for periods of about 1 week.)

Animal or group	Initial body weight (kg.)	No. of animals in group	No. of observations	Energy in food (kg. cal./day/sq.m.)	Total N retention (g.)	Total increase in body weight (kg.)		
						Calculated*	Observed	Calculated minus observed
114 (male)	13·90	—	1	1660	36·9	1·16	1·00	0·16
129 (female)	17·20	—	1	1665	38·8	1·22	1·05	0·17
138 (male)	15·90	—	1	910	16·7	0·53	0·50	0·03
141 (female)	12·00	—	1	1128	9·1	0·29	0·40	−0·11
All male animals	15·63	7	18	1186	28·0	0·875	0·740	0·135
All female animals	12·01	4	13	1340	20·6	0·644	0·519	0·125
All animals	14·11	11	31	1247	24·66	0·778	0·647	0·131 ±0·054†

* Calculation based on the assumption that the extra tissue contains an average of 3·17 % N (see p. 523).
† Standard error of mean value for 31 experiments.

the incorporation into the body of $\frac{12{\cdot}19 \times 3{\cdot}31}{100} = 0{\cdot}4035$ g. of N. The total amount of N fixed when 15 g. of body weight are added to 100 g. of normal dog under these conditions is thus $0{\cdot}0725 + 0{\cdot}4035 = 0{\cdot}4760$ g. The mean over-all N content of the added tissue is therefore $\frac{0{\cdot}4760 \times 100}{15} = 3{\cdot}17\%$. Since retention of electrolytes, bone and water probably occurred in addition to that of nitrogenous tissues, the figure of 3·17% N in the tissues added is likely to be a maximum. Nevertheless, it allows us to deduce a figure for the increase in body weight to be expected from the observed N retention if all the extra tissue has the composition of liver and muscle. Since the figure of 3·17% is probably a maximum, the expected increment in body weight calculated on this basis is likely to be minimal.

Table 3 gives a comparison of the observed increment in body weight, in 31 experiments, similar to that described in Fig. 2, together with the increment calculated as above from the observed retention of N. The data show that the mean calculated increment in body weight is close to that observed, but somewhat above it (Table 3), the difference between the calculated and observed values being statistically significant. Since the calculated increment is probably a minimum, the discrepancy may in reality be greater than is suggested by the data in Table 3. The results show, therefore, that the N retention resulting from pituitary treatment is certainly sufficient to account for the observed rise in body weight, so that there is no need to doubt that the animal acquires new tissue as the result of the pituitary treatment.

Energy rendered unavailable as the result of pituitary treatment. Before pituitary treatment our dogs were receiving a daily ration of food which was only just sufficient to maintain a steady body weight. As the result of pituitary treatment new tissue was built up, and, in most instances, sugar was also lost in the urine. Each of these processes resulted in a loss of oxidative energy, and before we consider how this energy might have been replaced we must assess its magnitude. Table 4 gives the results of calculations of the loss over the experimental period of about 1 week. The loss of calories resulting from the deposition of extra body tissue has been assessed on the calculated rise in body weight (see below) though the difference would not be very great if the figures were based on the rise actually observed (Table 3). The energy equivalent of the added tissues was calculated from the data in Table 2, from which it can be inferred that the retention of 1 g. of N entails the loss of 34 kg. cal. with the tissue thus added. As might be expected the total amount of energy lost to the animals which became diabetic as the result of pituitary treatment was greater than that becoming unavailable to animals which, for various reasons, resisted the diabetogenic action of the extract (Table 4). The mean result for the total of 31 experiments shows that 1033 kg. cal. were lost during the experimental period of approximately 1 week, so that, as the result of pituitary treatment under these conditions, 20·2% of the total energy intake could not be utilized.

Table 4. *Energy rendered unavailable in dogs as the result of anterior-pituitary treatment*

(Data given are mean values for periods of about 1 week.)

Group	No. of animals	No. of observations	Mean initial body weight (kg.)	Energy in food (kg. cal./day/sq.m.)	Total N retention (g.)	Energy rendered unavailable: kg. cal. By excretion of glucose	By increase in tissue	Total	Total as % of energy in food
Animals sensitive to diabetogenic action of extract	7	10	11·75	1318	26·40	463	898	1361	28·5
Animals not sensitive to diabetogenic action of extract	4	5	13·65	1171	30·56	0	1039	1039	21·6
All animals	11	31	14·11	1247	24·70	193	840	1033	20·2

Miscellaneous observations. It might well be assumed as the result of the above experiments that the metabolic rate of the meat-fed dog had been diminished as the result of pituitary treatment (cf. Young, 1942; Ogilvie, 1945; see also Teel & Cushing, 1930), and there are claims recorded in the literature that the metabolic rate of normal dogs is indeed diminished by treatment with pituitary extract (cf. Teel & Cushing, 1930). Nevertheless, the balance of the recorded evidence supports the conclusion that the metabolic rate is increased as the result of the administration of anterior pituitary extract to the intact dog (Gaebler, 1933; Houssay, 1934), though this is not necessarily true in all species (cf. Lee & Gagnon, 1930 for experiments on the rat).

Although data regarding the gaseous metabolism of dogs treated with diabetogenic pituitary extract

under the conditions of our experiments are not at present available, the indirect evidence which has been accumulated suggests that the metabolic rate is increased rather than diminished in dogs who receive a constant and limited diet of meat, gain weight and become diabetic as the result of the daily injection of diabetogenic anterior pituitary extract.

(*a*) *Body temperature and pulse rate.* In the two experiments on dog 140 (Fig. 1), observations showed that, although significant changes were not observed in most instances, both body temperature and pulse rate tended to be slightly elevated as the result of pituitary treatment (Table 5). Such a result would not be expected if the metabolic rate had been lowered by the treatment, and agrees with the idea that it was in fact raised.

Table 5. *Temperature and pulse records in anterior-pituitary treated dog* 140

(Data given are mean values (together with the standard errors of the means) for periods of about 1 week.)

Exp.		Pre-experimental control period	Experimental period	Post-experimental control period
a	a.m. temp.	101·1±0·16	102·3±0·20	101·5±0·29
	Difference		+1·2±0·26	−0·8±0·35
	a.m. pulse rate	130·6±1·87	132·0±1·11	130·0±0·12
	p.m. temp.	101·4±0·08	101·7±0·19	101·7±0·10
	p.m. pulse rate	128·7±2·64	130·3±0·80	134·3±1·40
b	a.m. temp.	101·0±0·18	101·5±0·17	101·0±0·24
	a.m. pulse rate	129·8±1·08	134·0±1·12	132·7±1·76
			+4·2±1·55	
	p.m. temp.	100·9±0·16	101·6±0·35	101·2
	p.m. pulse rate	132·7±1·98	132·0±5·66	128

(Only significant differences are indicated.)

(*b*) *Voluntary movements.* In the second experiment on dog 140 (Fig. 1; Table 5), voluntary movements (rockings of the cage) were recorded over three different control periods of 1 week, then during a week of daily injection of pituitary extract, and lastly for 1 week after the cessation of treatment. The three control periods gave mean results of 77·7±9·31, 85·0±15·9 and 76·5±9·08 movements of the cage daily. During the period of pituitary injection the recorded rockings averaged 69·8±10·12, and for the post-experimental control period the mean result was 136·4 ±13·61. It should be emphasized that during the various control periods the dogs were given daily injections of physiological saline so that no difference in handling or other treatment was associated with the institution of daily pituitary injections. It is clear that no significant change in the number of recorded voluntary movements resulted from the pituitary treatment, though it seems probable that movement was significantly stimulated by the cessation of pituitary treatment, i.e. substitution of saline for pituitary extract. Any diminution in metabolic rate might well have been reflected in a depression of voluntary movement on the part of the animal, and as no such depression was observed no evidence was thus forthcoming for a pituitary-induced diminution in metabolic rate.

It might here be mentioned that, like Benedict, Putnam & Teel (1930), we have observed that normal puppies treated daily with growth-promoting pituitary extracts over long periods of time become sluggish in comparison with control animals, and are definitely less inclined to undertake voluntary activity without good cause. The behaviour of the adult animals treated for shorter periods with a similar extract is, as indicated above, different from that of the puppies injected over a long period.

Replacement of the energy rendered unavailable by pituitary treatment. The experiments recorded above have shown that, in 31 experiments, the pituitary-treated dogs receiving a constant ration of food lost a total of 1033 kg. cal., on the average. This loss must be replaced by energy from a source not revealed by the analyses of which the results are given in Table 2. If fat from the fat depots were combusted to provide the necessary energy, 1033/9 = 115 g. (approx.) of this material would have to be oxidized, during the experimental period of approximately 1 week, if the total heat production were to remain unchanged. Now the results in Table 2 show that in the same 31 experiments the expected increase in body weight, calculated from the N retention, was, on the average, 131 g. more than that observed over the experimental period. If this discrepancy of 131 g. results from the oxidation of depot fat, then it follows from the considerations mentioned above that more than sufficient energy would be available for compensation of the loss resulting from the excretion of sugar and the assumption of extra liver and muscle tissue. Since the energy liberated by the oxidation of 1 g. of depot fat will be nearly nine times that which is rendered unavailable when 1 g. of normal muscle tissue is added to the body (Table 1), by combusting depot fat the animal can easily provide the oxidative energy required to maintain his metabolic rate, or even to increase it, while he is at the same time building up extra muscle and liver tissue to such an extent that the over-all effect on his body weight is augmentation (cf. Albright, 1942). This consideration also provides a reasonable explanation for the curious fact that the dog continues to retain N, during a period of pituitary-induced diabetes, at a time when the body weight is no longer rising (Figs. 1–3). The positive N balance may make material available for the deposition of extra muscle and liver tissue, the additional weight of which just compensates for the loss of body weight resulting from the oxidation of depot fat. It is on this basis that the increase in body weight calculated from the N retention, rather

than the increase actually observed, is utilized for the calculation of the energy rendered unavailable (p. 523).

Experimental verification of the deductions which emerge from the above argument would involve an estimation of the total fat content of the bodies of a large number of dogs, an undertaking which would be extremely difficult. The whole bodies of rats are more easily analyzed, but it is unfortunate that this animal differs from the dog in its response to the administration of diabetogenic pituitary extract in that the pituitary-treated rat fails to become diabetic but, like the puppy, grows instead. This is true, in our experience, for rats of all ages up to and including senility. Before experiments on rats were undertaken it seemed desirable, in the circumstances, to demonstrate findings, similar to those with the dog, on another species of animal susceptible to the diabetogenic action of the pituitary extract. Accordingly the experiments described above on dogs were repeated with intact adult cats.

B. Experiments on cats

Nitrogen retention and gain in body weight in pituitary-treated rats. Because of the fastidious taste of the cat it has been difficult to complete many experiments in which this animal ate a constant daily ration of food. Fig. 4 illustrates two experiments in which cats were given daily subcutaneous injections of 1 ml. of pituitary extract for 7 days. Unfortunately, in the second experiment (Fig. 4*b*), in which the pituitary treatment stimulated a substantial gain in weight despite the constancy of the daily food intake, the N balance of the animal was not determined.

In Table 6 are given the mean data for three experiments in which the N balance was determined, though the pituitary-induced gain in weight was, in each instance, unfortunately rather small. On the basis of the data in Table 6 calculation can be made, in a manner similar to that described for the dog (p. 523), of the energy rendered unavailable as the result of pituitary treatment. This is found to be 296 kg. cal., or 18·8 % of the energy intake, over the experimental period of approximately 1 week. The percentage datum is remarkably similar to that for comparable investigations on the dog, but, since there were so few experiments on the cat, the agreement may well be fortuitous. The amount of fat which a cat must oxidize if it is to replace this unavailable energy is approximately 33 g., and again, perhaps by good fortune, the discrepancy between the observed gain in weight and that calculated from the N retained is similar in magnitude, being 30 g. (Table 6). Although serious quantitative importance cannot be attached to these results, because only a few experiments were satisfactory technically, the data obtained certainly are not contrary to the view that the phenomena in the pituitary-treated cat receiving a constant daily amount of food are similar to those for the dog under similar conditions, and indicate that the animal probably oxidizes stored fat, while losing energy both as urinary glucose and as newly deposited muscle and liver tissues.

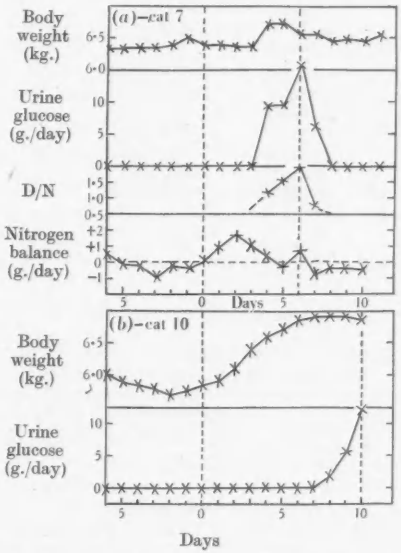

Fig. 4. Influence of daily injections of diabetogenic anterior pituitary extract on the normal cat. Throughout the experiments the animal consumed the same daily ration of meat food. This yielded, for cat 7, 860 kg. cal./day/sq.m. of initial body surface, and for cat 10, 1170 kg. cal./day/sq.m. of initial body surface. The first vertical dotted line (day 0) indicates the initiation of a series of daily subcutaneous injections of 1 ml. of pituitary extract. The second dotted line indicates the termination of injections.

Table 6. *Relationship between nitrogen retained, body weight gained and glucose excreted in cats under the influence of anterior-pituitary treatment*

(Data given are the mean values for periods of about 1 week.)

Initial body weight (kg.)	Total N retention (g.)	Total increase in body weight (kg.)		Total glucose excretion (g.)
		Calc.	Observed	
2·40	9·3	0·29	0·25	23·9
6·40	4·1	0·13	0·10	34·0
5·00	5·4	0·17	0·15	2·0
Mean 4·60	6·3	0·20	0·17	20·0
				34·2

Table 7. *Influence of anterior-pituitary treatment on the growth of rats receiving a constant daily amount of food*

Exp.	Group	Diet	No. of animals in group	Duration of exp. (days)	Body weight (g.) Initial	Final	Increase	Net increase (pituitary minus control)
				(*a*) Female animals				
1	Control	Constant	20	7	221·8	220·6	− 1·2	—
	Pituitary	Constant	20	7	220·6	252·1	+31·5	**32·7**
	Pituitary	Unlimited	20	7	226·7	270·4	+41·7	**42·9**
2	Control	Constant	10	21	210·5	223·6	+13·1	—
	Pituitary	Constant	10	21	216·8	274·9	+58·1	**45·0**
	Pituitary	Unlimited	10	21	223·4	303·5	+80·1	**67·0**
				(*b*) Male animals				
3	Control	Constant	10	21	127·3	138·0	+11·3	—
	Pituitary	Constant	10	21	131·8	172·6	+40·8	**29·5**

C. Experiments with rats

Lee & Schaffer (1934) found that pituitary-treated intact rats limited to the same intake of food as control animals ('paired-feeding control' experiments) gained significantly more weight than did the control rats. Marx, Simpson, Reinhardt & Evans (1942) obtained similar results when hypophysectomized pituitary-treated rats were compared with untreated hypophysectomized animals in similar 'paired-feeding control' experiments. The experiments we have carried out with rats were designed to be as comparable as possible with those on the dog and the cat described above, so our rats were trained or constrained to eat a constant daily ration of food (meat) which was just sufficient, in the absence of pituitary treatment, to maintain a constant body weight. Thus our experiments differed from 'paired-feeding control' experiments in that, although the control and experimental animals consumed a similar daily ration of food, this was not the maximum amount which the animals in one of the groups would voluntarily eat and which varied from day to day.

Rise in body weight of pituitary-treated rats receiving a constant daily ration of food. As with dogs and cats, pituitary treatment of the rat induced a substantial rise in body weight despite the limitation of food intake (Table 7). This was true for adult female rats, whose growth curves had reached the period of 'plateau' and who were therefore gaining only 1–2 g./week under normal conditions, and also for young growing male rats (Table 7). Since the food ration of the young animals was so adjusted as to maintain an approximately constant body weight before pituitary injections were begun these animals were consuming much less food than was normally required to support growth, but nevertheless they gained markedly in weight as the result of the daily administration of the pituitary extract (Table 7).

The data given in Table 7 relate to experiments lasting 1 or 3 weeks but one of these experiments ((*a*) 2, Table 7) was continued over a total period of 9 weeks. The results of this prolonged treatment (Fig. 5) show that although the rise in body weight

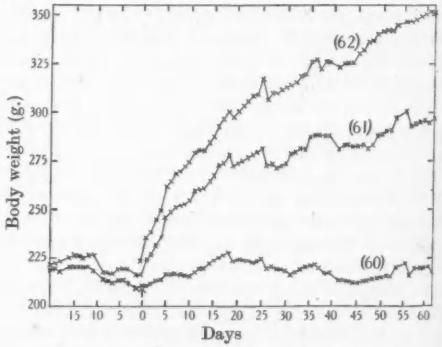

Fig. 5. Influence of daily injections of anterior pituitary extract on the growth of the adult female rat. Each group contained ten animals, each animal being maintained in a separate cage. The rats in groups 60 and 61 were given and consumed the same daily ration of meat food throughout the period illustrated; this ration provided 52 kg. cal./day or approx. 1800 kg. cal./day/sq.m. of initial body surface. The animals in group 62 ate *ad libitum* and their mean intake during the period of daily pituitary injections was 42% above that of the rats in groups 60 and 61. On day 0, and thereafter until the end of the period illustrated, each rat in groups 61 and 62 received daily a subcutaneous injection of 0·5 ml. of anterior pituitary extract.

in the pituitary-treated rats receiving a fixed diet was rapid for the first 3 weeks of treatment and approached that of the treated animals allowed to eat *ad libitum* (cf. Table 7), the rise of the former was subsequently less so that the difference between

the pituitary-treated rats allowed to eat *ad libitum* and those receiving a fixed ration was much greater after 9 weeks of treatment than after 3. Nevertheless, the animals receiving the fixed ration gained 80 g. on the average as the result of pituitary treatment, an increase amounting to 37 % of their initial weight. The pituitary-treated animals receiving unlimited diet gained 133 g., or 60 % of their initial weight.

Table 8. *Dimensions of control and pituitary-treated rats*

	Group no. 60 (Control rats—constant diet)	61 (Pituitary-treated rats—constant diet)	62 (Pituitary-treated rats—unlimited diet)
Weight (g.):			
Initial	210·5	216·8	223·4
Final	217·7	297·1	356·2
Gain	7·2	80·3	132·8
Net gain	—	73·1	125·6
% net gain	—	**33·7**	**56·2**
Body length (mm.):			
Final	191·6	201·0	207·8
Net gain	—	9·4	16·2
% net gain	—	**4·9**	**8·5**
Tibia length (mm.):			
Final	36·6	39·4	38·8
Net gain	—	2·8	2·2
% net gain	—	**7·6**	**6·0**
Tail length (mm.):			
Final	178·5	183·4	184·6
Net gain	—	4·9	6·1
% net gain	—	**2·7**	**3·4**
Surface area (sq.cm.):			
Final	285	306	352
Net gain	—	21	67
% net gain	—	**7·4**	**23·5**
Cross-section (sq.cm.):			
Final	11·03	14·05	16·49
Net gain	—	4·02	5·46
% net gain	—	**36·4**	**49·4**
Volume (ml.):			
Final	211·4	282·4	342·8
Net gain	—	71·0	131·4
% net gain	—	**33·6**	**62·2**
Body weight/body length (g./mm.):			
Final	1·14	1·48	1·72
Increment*	—	**8·45**	**8·55**
Body weight/tibia length (g./mm.):			
Final	5·94	7·54	9·18
Increment	—	**28·4**	**63·0**
Body weight/tail length (g./mm.):			
Final	1·22	1·62	1·94
Increment	—	**16·20**	**22·70**
Body weight/surface area (g./sq.cm.):			
Final	0·762	0·971	1·012
Increment	—	**3·775**	**2·062**
Body weight/cross-section (g./sq.cm.):			
Final	19·7	21·2	21·6
Increment	—	**19·7**	**25·4**
Specific gravity:			
Final	1·030	1·052	1·039
Increment	—	**1·118**	**1·054**
Nutritive index†:			
Final	0·313	0·336	0·341

* I.e. increase in body weight (g.) divided by increase in body length (mm.). In this connexion the increase in weight is considered to be the difference between the final weights of the experimental and control groups.

† $w^{\frac{1}{3}}/l$ (Cowgill & Drabkin, 1927), where w = body weight in g. and l = body length (tip of nose to anus) in cm.

Dimensions and composition of the pituitary-treated rats. In the experiment described above, which lasted 9 weeks, the animals were killed at the end of this period, and the data in Tables 8–10 obtained by measurement and analysis. Table 8 shows that the configuration of the pituitary-treated animals differed significantly from that of the control rats, and that the rise in body weight was accompanied by only a small increase in the length of the body, of the long bones (tibia) and of the tail, while the mean area of cross-section of the body (volume/length), and body volume, were both substantially increased. The increase in volume was not, however, proportional to the rise in weight, and the mean specific gravity of the injected rats was high, the specific gravity of the acquired material being 1·118 for the pituitary-treated rats receiving a constant food intake (Table 8).

The elongation of the tibiae of the pituitary-treated rats with constant food intake was greater than that of the treated rats given unlimited food, although the increment in body weight and body length of the latter group was the greater. The treated animals on limited food intake also exceeded their unrestricted sisters in specific gravity.

Determination of the mean composition of the whole bodies (Table 9) indicated the reason for the rise in specific gravity of the whole animal which followed pituitary treatment, since it was found that the treated rats had a higher content of water and protein, and a lower content of fat, than did the

Table 9. *Composition of control and anterior-pituitary treated rats*

Content of	Mean composition (%) of animals in group no. 60 (Control rats—constant diet)	62 (Pituitary-treated rats—unlimited diet)	61 (Pituitary-treated rats—constant diet)
Protein	16·80	17·70	18·70
Fat	15·45	9·12	7·15
Carbohydrate	4·08	2·10	2·56
Ash	4·21	3·18	4·61
Water	59·44	67·90	66·98
Solid	32·40	27·90	27·90
Dry fat-free solid	25·11	22·98	25·87
Energy (kg. cal./100 g.)	223	161	149

control animals. In experiments concerning the influence of growth-promoting pituitary extracts on the growth and composition of the intact rat, Lee & Schaffer (1934) showed that pituitary treatment induces a rise in protein and water content of the carcass, with a diminution in fat content. Our pituitary-treated rats receiving a constant amount of food daily throughout the 9-week experimental period exhibited a greater rise in protein content, and a larger diminution in fat content, than did the treated rats allowed to eat *ad libitum*. The restriction of food intake had apparently exaggerated the changes in body composition which normally follow pituitary treatment in the rat.

Photographs of representative pairs of rats from the three groups (Fig. 6) show that the pituitary-treated animals with constant food intake appeared both bigger and better nourished than did the controls consuming the same food ration. The well-nourished appearance is associated with a higher 'Nutritive Index' (Cowgill & Drabkin, 1927) than that of the control rats, a fact which is of particular importance in view of the fact that the percentage of body-fat (ether-soluble material) in the pituitary animals was less than one-half that of the controls receiving the same amount of food (Table 8). Thus with these animals the Nutritive Index is misleading and the true state of affairs is most easily revealed by the rise in the mean specific gravity of the body tissues (Table 8). Such was almost certainly true for the pituitary-treated dogs, which appeared exceedingly well-nourished as the result of pituitary injection (p. 522), but unfortunately no measurements of specific gravity or of Nutritive Index were made with the dogs.

Energy rendered unavailable by pituitary treatment. The rats with a constant intake of food gained a substantial amount of body weight as the result of pituitary treatment, but because of the hypertrophy of nitrogenous tissues, associated with a fall in fat content, the total energy content of the whole body sharply diminished (Table 10, cf. Lee & Schaffer, 1934), as also did the energy content/100 g. of tissue (Table 9). On the other hand, the animals allowed to eat *ad libitum* during the pituitary treatment gained substantially in total stored energy as the result of treatment (Table 10) though the energy equivalence of 100 g. of tissue diminished (Table 9). As was emphasized above, the intact rat does not become diabetic as the result of pituitary treatment and no energy was lost, therefore, in the form of urinary sugar.

The animals in receipt of a constant ration of food acquired 19·2 g. of protein, 69·8 g. of water and 4·56 g. of ash (total 93·6 g.) as the result of pituitary treatment (Table 10), at the same time losing 12·3 g. of fat and 1·27 g. of carbohydrate, etc. If we express, in the form of a percentage of the gross weight of matter added, the amount of each substance deposited as the result of pituitary treatment, it appears that the additional material contained 20·45% of protein and 74·5% of water; this is similar to the composition of muscle tissue. The alterations in liver size as the result of pituitary treatment in these experiments were relatively small, being 4·70% of the total gain for the pituitary-treated animals on the fixed diet, and 9·12% for those on an unlimited diet. The livers were not analyzed separately, but it seems probable that no substantial error will be introduced if we assume that the whole of the material added to the bodies of the pituitary-treated rats possessed a composition similar to that of normal muscle, i.e. it contained 21·2% protein, 2·5% fat, 1% of carbohydrate, etc., 1% of ash and 73·2% of water. If this assumption is made, then the 19·2 g. of protein deposited represents the addition of 90·2 g. of tissue and a loss of available energy of 101 kg. cal. The 12·3 g. of fat lost from the whole body (Table 10) represented 110·7 kg. cal. of available energy, an amount which is approximately equal to that lost by the addition of new tissue (101 kg. cal.).

Table 10. *Composition of material added to the bodies of rats as the result of pituitary treatment*

Total amount added of: Protein (g.)	Fat (g.)	Carbohydrate (g.)	Ash (g.)	Water (g.)	Solid (g.)	Energy (kg. cal.)
(*a*) In the 80·3 g. added to rats in group 61 (constant diet)						
+19·2	−12·3	−1·27	+4·56	+ 69·8	+10·19	−39
(*b*) In the 132·8 g. added to rats in group 62 (unlimited diet)						
+25·6	− 2·0	−1·64	+1·94	+105·7	+23·90	+78

It is obvious that in view of the many possible errors attached to the elicitation of the data employed in these calculations no significance can be attached to the difference between the caloric equivalence of the fat which disappears (110·7) and the calories entrained by the deposition of tissue in the rats on a constant food intake (101·0), but it may be pointed out that the discrepancy observed would be in keeping with the existence of a rise in metabolic rate as the result of pituitary treatment. The difference is relatively small, however, and the results indicate that, as was deduced to be the case with the dog and the cat, the pituitary-treated rat receiving a fixed amount of food daily apparently deposits nitrogenous tissue, of the composition of muscle substance, at the expense of the oxidation of an amount of fat which is approximately equicaloric, the over-all effect on the body weight being a substantial rise. With the pituitary-treated rats allowed to eat *ad libitum*, calculation shows that about 121 g. of material with the composition of muscle tissue were deposited, which rendered unavailable 134 kg. cal. of energy. These animals lost only 2 g. of total body fat (Table 10), which would make available 18 kg. cal. of energy. The rest of the energy debt (116 kg. cal.) resulting from the deposition of tissue is undoubtedly made good from that available as the result of the consumption of extra food. The extra food actually consumed provided more energy, amounting to an average of 22·5 kg. cal./day above that accessible to the control animals; thus over the whole period of pituitary treatment an increment of nearly 1400 kg. cal. was available—over ten times the amount required to balance the calorie budget if the metabolic rate in terms of total calories per day remains unaltered from that obtaining during the control period. It is clear that there is ample energy available from the extra food to allow for a rise in metabolic rate in the pituitary-treated rats receiving an unlimited amount of food.

Changes in metabolic rate resulting from the administration of pituitary extract. The calculation of the metabolic rate from the amount of food consumed is valid only if all the available combustible foodstuff is actually burned during the period under consideration, the composition of the body alters in no way, and the body does not change in weight. None of these criteria is satisfied in the experiments with the pituitary-treated rats receiving either a constant daily amount of food or an unlimited diet, since the body weight and body composition changed as the result of treatment. If, nevertheless, we calculate the apparent metabolic rate from the observed food intake over the last 2 weeks of the experiment, we find that the mean daily absolute calorie intakes were 52 kg. cal. for the control and pituitary-treated animals on a fixed ration, and 80 kg. cal. for the pituitary-treated animals receiving unlimited food. For the expression of these metabolic rates in terms of calorie intake per sq.m. of surface area, the surface area was deduced from the mean weight of the animals during the last 2 weeks of the experiment. Calculated thus the metabolic rate of the control animals is 1810 kg. cal./day/sq.m. of body surface, while for the pituitary-treated groups on fixed diet and on unlimited diet the values were 1690 and 2260 kg. cal./day/sq.m. respectively. The calculated values for the pituitary-treated animals on a fixed diet must be too low, since these animals were losing calories from their tissues despite the increase in body weight (Table 10); while that for the pituitary-treated rats in receipt of unlimited food is too high since, although the energy content of their tissues per 100 g. of body weight was diminished as the result of the injections of pituitary extract (Table 9), the total energy content of the (enlarged) body was greater at the end of the period of pituitary injections than it was calculated to have been at the beginning of the experiment (Table 10). But although some calories had been absorbed in the tissues of the treated animals receiving unlimited food, this loss of oxidative energy was probably quantitatively insignificant over the whole experimental period. Nevertheless, it was to be expected that the metabolic rate of the animals who were allowed to eat unlimited food, and whose appetites were increased as the result of pituitary treatment, would be exalted as the result of the specific dynamic action of the extra protein absorbed, and the result of the calculation above agrees with this expectation.

Although no direct observations on the metabolic rates of the treated rats were available, indirect evidence suggested that pituitary treatment had increased the expenditure of energy by these animals.

(*a*) *Body temperature.* In the experiments of which some results are given in Fig. 5 and Tables 8–10, rectal temperatures were recorded for 3 days before pituitary injections began, and for 6 days after. The mean pre-injection body temperature for the animals in all three groups was 97·9 $\pm$0·11° F., where 0·11 is the standard error for the mean result for a group of 30 rats. In the control animals, given daily injections of saline, the mean rectal temperature over a period of 6 days was 97·7$\pm$0·11° F., while the corresponding figures for the pituitary-treated groups were 97·9$\pm$0·08° F. (constant food intake) and 98·1$\pm$0·16° F. (unlimited food intake). It is clear that no diminution in body temperature occurred of the type that might be expected if the metabolic rate fell as the result of pituitary treatment, but, although the body temperature tended to rise as the result of the treatment, the changes are not statistically significant on the basis of the number of observations recorded.

(*b*) *Voluntary movements of pituitary-treated rats.* A fall in available energy as the result of pituitary treatment might be reflected in a diminution in the spontaneous activity of the animal. Two experiments were therefore carried out concerning the number of times each day a pituitary-treated rat would voluntarily revolve a cage. In the first experiment, lasting 10 weeks, a rat consuming an uncontrolled amount of food was given daily subcutaneous injections of 0·5 ml. of anterior pituitary extract, or 0·5 ml. of saline, during alternate weeks. The mean number of revolutions turned per day during the five control weekly periods was 1299, while for the five periods of 1 week during which pituitary extract was injected daily the mean number of revolutions was 1176/day. Thus in this preliminary experiment the pituitary treatment appeared to exert no significant influence on the voluntary exercise undertaken

by the animal when the animal was allowed to eat *ad libitum*.

Throughout the second experiment a rat was given a fixed ration (meat diet) which supplied 1669 kg./cal./day/sq.m., and after a control period of 2 weeks with daily injections of 0·5 ml. of saline the animal received 0·5 ml. of anterior pituitary extract daily for 8 weeks, followed by saline for a post-injection control period of 1 week. From the following results it is seen that pituitary treatment rendered this lively rat even more lively, in spite of the restricted daily ration of food, and despite the increase in body weight with consequent loss of potential calories.

	Mean body weight (g.)	Mean no. of revolutions turned per day
Pre-injection control period	224·3	3206
Period of pituitary injections	229·5	5111
Post-injection control period	221·4	5564

A more precise determination of the effect of the pituitary extract would have involved longer experiments with large numbers of trained rats, but since these preliminary results suggested that no significant diminution in spontaneous activity resulted from pituitary treatment at a time when the animal was receiving an amount of food only just sufficient, under normal conditions, to maintain a constant body weight, no further investigations were undertaken in this connexion.

DISCUSSION

Nature of the body tissue acquired as the result of pituitary treatment. Our results show that an important action of diabetogenic anterior pituitary extract is the preservation from oxidation, with consequent retention in the body, of protein, with a simultaneous enhancement of the combustion of fat. They agree with the findings of others regarding the influence on metabolism of hypophysectomy and of treatment of normal animals with pituitary growth-hormone preparations. Lee & Schaffer (1934) showed that the pituitary-treated intact rats contained less fat and more N than 'paired-fed' control animals. Lee & Ayres (1936) (see also Schaffer & Lee, 1935) further found that, when normal rats were restricted to the same low food intake as that voluntarily consumed by hypophysectomized litter-mate animals, the normal rats lost 60 % of their initial fat content in 33 days although they retained their original body N. The hypophysectomized rats, on the other hand, lost only 28 % of their initial fat stores but failed to retain 19 % of their original N, the over-all effect in the body weight being a loss which was 20 % greater than that in the 'paired-fed' control animals. A corollary of this is seen in the experiments of Samuels, Reinecke & Bauman (1943), who found that when young hypophysectomized rats are forcibly fed an amount of food sufficient to induce growth in normal litter-mate control animals, they stored more fat and less protein than the intact control animals, the over-all increase in body weight of the hypophysectomized rats being much less than that of the controls. Comparable results have been obtained by Levin (1944).

Since the specific gravity of fat is less than unity, while that of protein-containing tissue, with a substantial percentage of water, is above one, the exchange of fat and nitrogenous tissue led to a rise in specific gravity of the body mass in our experiments (rat—Table 8). Thus an increase in body weight, associated with an appearance of plumpness and a rise in the Nutritive Index (Table 8), may be associated with a diminution in the total fat content of the body. In such cases the determination of the specific gravity of the body becomes of importance, and it is interesting to note that Behnke, Feen & Welham (1942) and Welham & Behnke (1942) have recently determined the specific gravity of healthy men, and have proposed that classification as overweight should be based on specific gravity of the body mass rather than on the quotient weight/height, which is the usual criterion. A determination of the corporeal specific gravity in the over-weight condition of certain pathological states (e.g. anterior pituitary hyperfunction) might be of value, and in view of the present results it would be of great interest to know whether or not the over-weightness of elderly people who ultimately become diabetic (Joslin, 1940) is always associated with a fall in specific gravity, since such should be the case if the extra weight were largely the result of the deposition of fat. If experimental results regarding the diabetogenic action of the anterior pituitary gland are relevant to the question of the aetiology of diabetes in the elderly over-weight person (cf. Young, 1941, 1944), the possibility can be considered that, with the development of hyperactivity of the anterior pituitary lobe in a fully grown human being, the body weight might rise as the result of the deposition of protein-containing tissue and simultaneous enhancement of the rate of oxidation of the fat stores even though the food consumption were not increased. Such a state of affairs could be revealed most easily by a determination of the mean specific gravity of the body tissues, since in such circumstances it should rise, while it would fall in a person who became over-weight as the result of adiposity.

Growth in pituitary-treated animals. That the anterior pituitary lobe controls the growth of somatic tissues is certain. Nevertheless it is clear that in normal growing animals the extra tissue deposited under the influence of pituitary treatment differs in composition from that which would accrue without administration of the extract. As Lee & Schaffer (1934) point out, their pituitary-treated normal rats retained their original composition almost precisely, whereas the control animals exhibited changes

normally associated with growth—e.g. a diminution in the percentage contents of N and of water, and an increase in the percentage of fat. The results of the present investigation agree with the belief that treatment with anterior pituitary extract of normal animals allowed to eat freely leads to an increase in body weight largely by inducing a replacement of fat by protein-containing tissue, the latter possessing greater mass per unit of contained energy than does deposited fat. This replacement is associated with a rise in appetite, an elongation of the skeleton and a general increase in weight of the tissues of the body. Such a process undoubtedly ranks as true growth. On the other hand, when the food consumption of the animal is limited to that which would just maintain a steady weight before pituitary treatment began, the daily injection of anterior pituitary extract leads to similar but more exaggerated changes in body composition (cf. Table 9). Under these conditions there occurs an interchange of body constituents with those of the food, with some over-all loss of energy content of the enlarged body mass. Fat is lost and partly replaced, calorically, by protein-containing tissues possessing more than 70% of water. The result is an increase in body weight and volume and a diminution in total energy content. Does such a change qualify for the description of growth? In the absence of a precise definition of this term the answer may be given, with perhaps some misgiving, that there is no obvious reason why this process should not be called growth, particularly since the skeleton elongates, albeit little (Table 8). In the dog the weight gained under the influence of pituitary treatment may be lost, in part or wholly, after the daily injections of extract cease, if treatment has not been prolonged. With long periods of daily injection the extra body weight appears to be retained in part after treatment ceases (cf. Fig. 3).

Although the extra nitrogenous tissues acquired as the result of pituitary treatment may be similar in composition to normal muscle there was little evidence of an abnormal increase in functional muscular tissue in our pituitary-treated animals. Plattener & Reed (1939) found that the muscles of pituitary-treated normal rats were larger than those of control animals but that the treated animals gained no physiological advantage therefrom. Pituitary treatment of the intact animal results in a generalized deposition of protein-containing tissue (cf. Schaffer & Lee, 1935), particularly in and around the abdomen, a place where it fulfils no obvious mechanical purpose.

We may conclude that, contrary to the opinion of some workers, pituitary-induced growth does not result from an increased efficiency of utilization of foodstuffs; at least it certainly does not in all cases.

Mechanism of the acquisition of extra body tissue under the influence of pituitary treatment. In the animal lacking a pituitary gland and starving, or receiving food containing little or no protein, the metabolism of endogenous protein is significantly less than in the normal animal under similar dietary conditions (dog—Braier, 1931; rat—Braier & Morea, 1935, and Fry & Long, 1943), while liver arginase activity is diminished in the hypophysectomized rat (Fraenkel-Conrat, Simpson & Evans, 1943). The diminished protein catabolism of the hypophysectomized animal may be raised by the administration of an anterior pituitary preparation (dog and rat—Braier & Morea, 1936), while the liver arginase activity of the hypophysectomized rat is also enhanced as the result of suitable pituitary treatment (Fraenkel-Conrat *et al.* 1943). The effect of hypophysectomy in lowering endogenous protein catabolism may result from a diminished secretory activity of the adrenal cortex in the absence of pituitary stimulation (cf. Fry & Long, 1943; Fraenkel-Conrat *et al.* 1943).

In contrast with the metabolism of endogenous protein, that of exogenous protein is enhanced by hypophysectomy (rat—Schaffer & Lee, 1935; Lee & Ayres, 1936; Perla & Sandberg, 1936; Samuels *et al.* 1943). Although they found that in the hypophysectomized rat or dog the excretion of N after a meat meal was slow, but normal in total amount, Braier & Morea (1935) did not determine the mean daily N balance over a period of time, and their results do not seriously contradict other demonstrations that the storage of exogenous protein is diminished, and its catabolism enhanced, as the result of hypophysectomy. Therefore, after hypophysectomy, the diminution of endogenous protein catabolism is associated with a depressed ability to incorporate exogenous protein (or rather amino-acids) into the tissues. Conversely, there is evidence that factors which normally tend to stimulate the catabolism of endogenous protein may, under some conditions, stimulate the retention of protein-containing tissue in the body. Thus, despite the fact that the administration of adrenal cortical extract normally enhances protein catabolism (Ingle, 1944), the presence of the adrenal cortex or of its secretions appears to be essential for the manifestation of the full growth-promoting activity of anterior pituitary extract (rat—Fry & Long, 1943). Again, although thyroxine is normally regarded as a stimulator of protein catabolism, the cretin does not retain N and grow unless treated with thyroid-active material. Furthermore, treatment with small doses of thyroid-active substances may induce N retention and growth in normal animals (human—Johnston & Maroney, 1939; mouse—Kroger, Hurst & Turner, 1942), and can act synergistically with pituitary extract in stimulating growth in hypophysecto-

mized-thyroidectomized or normal rats (Smith, 1933; Evans, Simpson & Pencharz, 1939). The demonstration of a synergism between pituitary thyrotropin and growth hormone in the hypophysectomized rat by Marx, Simpson & Evans (1942), and the observation of Gaebler & Robinson (1942) that thyroidectomy diminishes the N-retaining action of anterior pituitary extracts in the dog under some conditions, are also relevant here.

In the light of the above facts it seems probable that the incorporation of exogenous amino-acids into the tissues is assisted by a limited stimulation of the catabolism of tissue (endogenous) protein. The over-all result of an increase in the rate of amino-acid turnover may be the retention, in the tissues, of more protein than was previously present there. Such a view is not inconsistent with the demonstration that treatment with pituitary adrenocorticotropin (Marx, Simpson, Li & Evans, 1943; Becks, Simpson, Marx, Li & Evans, 1944) or with adrenal-cortical hormones (Wells & Kendall, 1940; Ingle, 1941, 1944), may inhibit growth under some conditions, since it is obvious that the balance between protein catabolism and anabolism will be upset by undue emphasis of the catabolic side.

If this view is correct there must exist in the body a pituitary-stimulated mechanism which enhances protein anabolism. The secretion of insulin is capable of fulfilling such a mechanism (Janney & Shapiro, 1926; Mirsky, 1938, 1939; MacKay, Barnes & Bergman, 1939) provided that, under its influence, the blood sugar is not allowed to fall to hypoglycaemic levels, but is maintained or even elevated. The importance of preventing hypoglycaemia in studies of the physiological action of insulin has recently been well emphasized by de Duve (1945).

Mirsky (1939), Young (1939, 1941, 1944), Gaebler & Galbraith (1941), Gaebler & Robinson (1942) have all provided evidence that the influence of anterior pituitary extract in inducing N retention is mediated, at least in part, by the secretion of pancreatic insulin. Gaebler & Robinson (1942), however, were able to bring about some N retention, albeit slight and inconsistent, by the administration of a preparation of prolactin to the depancreatized dog receiving a constant daily dose of insulin; one pituitary fraction therefore appears to be capable of inducing N retention in the absence of extra insulin. But other pituitary preparations induce N loss unless excess of insulin is simultaneously made available (Mirsky, 1939; Gaebler & Galbraith, 1941). Accordingly pituitary-induced growth can occur only if the islets of Langerhans of the pancreas are capable of furnishing enough insulin to support it. If pancreatic insulin be relatively deficient, pituitary treatment may induce little or no positive N balance, and, indeed, in extreme cases the balance may become negative as the result of such treatment (Young, 1941; Gaebler & Galbraith, 1941). Under these conditions glycosuria will be induced or enhanced by the administration of the pituitary extract.

Relationship between the growth-promoting and diabetogenic activities of anterior pituitary extract. Although preparations of pituitary growth hormone can be obtained which do not produce diabetes in susceptible intact animals (Marks & Young, 1940), yet, in our experience, growth-promoting pituitary extracts are always diabetauxetic (Young, 1941), i.e. their administration exacerbates an existing severe diabetes. It is therefore of particular interest that recently Marx, Anderson, Fong & Evans (1943) have shown that their purified preparation of pituitary growth hormone induces a significant increase in glucose excretion in the sucrose-fed, partially depancreatized rat. In the hypophysectomized rat with intact pancreas such pituitary growth-promoting preparations lower the blood sugar level (Marx, Herring & Evans, 1944; cf. also Horsters, 1933, and Weinstein, 1939) and show high myoglycostatic activity (Herring & Evans, 1943), i.e. they are active in retarding the rapid oxidation of the carbohydrate stores of the muscles which characterizes the fasting hypophysectomized rat.

It is our belief that growth-promoting pituitary preparations are essentially myoglycostatic and diabetauxetic, their action being intimately concerned with a depression of carbohydrate oxidation and with the ability of insulin, secreted by the islets of Langerhans of the pancreas, to aid the incorporation of blood amino-acids into tissue protein. Thus the over-all effect of pituitary treatment on the metabolic pattern of an animal receiving protein-containing food is a depression of oxidation of carbohydrate and of protein, associated with an increased rate of combustion of fat. Under these circumstances the fat stores of the body may be in part replenished by the conversion of carbohydrate to fat, a conversion which is probably under the influence of the secreted insulin (cf. Drury, 1940).

It must be admitted that diabetogenesis is not a normal physiological function of the anterior pituitary lobe, and that such a pathological process as the development of pituitary-induced diabetes may be the result of an inappropriate enhancement of a normal activity of the gland. As we have seen above, the administration of anterior pituitary preparations can lead, in the normal or in the hypophysectomized animal receiving protein-containing food, to a depression of oxidation of protein and of carbohydrate. It is an uncontrolled stimulation of this pituitary activity that leads to the diabetogenic action of anterior pituitary extracts in intact animals.

In an animal which possesses normally functioning pancreatic islets it seems probable that

pituitary diabetogenic activity cannot be made manifest until insulin production by the pancreas has become relatively deficient. Approximate calculations, based on the results of Best, Campbell, Haist & Ham (1942), and of Young (1944), suggest that during the early days of treatment with diabetogenic pituitary extract the dog with pancreas intact is producing each day 10–20 times the normal amount of insulin (Young, 1944). While the islets retain the capacity to secrete insulin in such large amounts the conditions obtaining are those which may be expected to lead to the retention of N, namely, enhanced secretion of insulin, associated with maintenance of the blood sugar at normal or hyperglycaemic levels. The normal pancreas possesses a substantial capacity for the production of extra insulin, and a reduction of this capacity under pituitary influence can apparently occur only after a preliminary excessive outpouring of insulin, possibly in response to a pituitary-induced lowering of the concentration of insulin in the blood. Houssay, Foglia, Smyth, Rietti & Houssay (1942) believe that diabetogenic pituitary extract exerts a direct damaging action on the pancreatic islets, but whether or not this is so it is certain that pituitary diabetogenesis must be preceded by, and in part accompanied by, an excessive secretion of pancreatic insulin perhaps associated with islet hypertrophy (Richardson & Young, 1938; Ogilvie, 1945), with concomitant N retention and enhancement of the weight and bulk of the tissues of the body.

In the present experiments with normal dogs N retention was observed even when a substantial pituitary-induced glycosuria was manifest. Similarly, in experiments with depancreatized dogs maintained with insulin, Gaebler & Galbraith (1941) and Gaebler & Robinson (1942) observed that N retention, induced by simultaneous administration of pituitary extract and extra insulin, was frequently accompanied by a rise in glycosuria. Thus even when the amount of insulin secreted by the pancreatic islets under pituitary influence is insufficient to neutralize the depression of carbohydrate oxidation brought about by the pituitary extract, the insulin is still able to stimulate protein anabolism, annulling any catabolic influence on protein metabolism directly exerted by pituitary factors (cf. Paschkis, 1942). The reason is therefore clear why, in our experiments with intact dogs, the diabetogenic pituitary extract always initially exerted a substantial N-retaining action, some part of which was usually sustained so long as the diabetogenic action of the extract was manifest. As might be expected, however, if effective diabetogenic treatment is prolonged until extensive islet degeneration occurs and the temporary diabetic condition is converted into a permanent one, N balance becomes negative and body weight is lost (Young, unpublished observations; see also Young, 1941). In such a case N retention and a rise in body weight can be induced if insulin is given simultaneously with the diabetogenic pituitary extract (Young, 1944, 1946).

It is significant that when a dog became resistant to the diabetogenic action of the pituitary extract he also ceased to retain N and to gain weight under its influence (cf. Fig. 3). It seems unlikely, therefore, that such developed resistance to the diabetogenic action of the extract can result only from islet hypertrophy (Young, 1940) and it must be ascribed in part to adaptation of the animal to the excessive pituitary stimulus, either by antihormone production (cf. Dohan & Lukens, 1939) or by other at present undetermined means. But the fact that diabetogenic and N-retaining activities disappeared together is an important corollary of the above argument.

The argument leads to the conclusion that a diabetic condition is the pathological outcome of pituitary-stimulated growth in an animal whose pancreatic islets become unable to secrete enough insulin to prevent the serious depression of carbohydrate oxidation resulting from the action of pituitary factors. For the first 2 or 3 days of pituitary treatment insulin secretion is sufficient, even in a susceptible intact animal such as the dog or cat, to maintain carbohydrate oxidation at a rate which prevents the development of hyperglycaemia under the conditions obtaining; hence the diabetogenic action of the extract develops, in the intact dog and cat, only after a characteristic latent period of some days, during which substantial N retention occurs. In a species resistant to pituitary diabetogenic action, such as the normal intact rat, the extra insulin secretion by the animal's robust pancreatic islets is apparently sufficient to maintain carbohydrate oxidation at a rate compatible with a normal blood-sugar level. Hence in such an animal the predominant action of the pituitary extract is a 'pancreatropic' one (Richardson & Young, 1937; Marks & Young, 1940).

Since other pituitary factors act synergistically with the growth-hormone of H. M. Evans, the presence of this hormone cannot quantitatively account for the full growth-promoting activity of a crude pituitary extract. It is possible that a 'growth-complex', responsible for the full growth-promoting activity of a crude pituitary extract, is identical with a 'diabetogenic complex', but since the diabetogenic activity (for the intact animal) of an anterior pituitary extract is easily lost, while the recognized pituitary factors which influence metabolism are not markedly unstable, it seems possible that the 'diabetogenic-complex' cannot yet be completely characterized. Whether or not the factors comprising the pituitary 'growth complex' or 'diabetogenic complex' are present in the gland

in combination will be the subject of another paper.

The D/N quotient in pituitary-induced diabetes. The high corrected D/N quotients encountered in the present investigation are of particular interest. Quotients of a similar magnitude have been observed by Lewis, Kuhlman, Delbue, Koepf & Thorn (1940) in phlorrhizinized rats treated with adrenal-cortical extract, and by Ingle & Thorn (1941) in partially depancreatized—and in partially depancreatized-adrenalectomized—rats treated with 17-hydroxy-11-dehydrocorticosterone. In our pituitary-treated dogs activity of the adrenal cortex was almost certainly enhanced, and this enhancement may be associated with the high D/N quotients, but the metabolic reason for such high quotients is not clear. The positive N balance obtaining during the period when these quotients were observed should not have been responsible for their highness since, in accordance with accepted theory, retention of N to form new tissue should be associated with the retention of an equivalent amount of carbohydrate, the D/N quotient remaining unaffected. In earlier experiments (Young, 1941), in which the administration of non-diabetogenic pituitary extract to a mildly diabetic dog induced N retention, the D/N quotient simultaneously fell, presumably owing to the predominant action of insulin in promoting carbohydrate oxidation.

The diabetogenic pituitary extract depresses carbohydrate oxidation, but its administration to a normal animal could be expected to result in a D/N quotient above that of the depancreatized dog only if it be assumed that carbohydrate oxidation is occurring in the fully depancreatized animal or that the pituitary extract stimulates sugar formation from some non-protein source. A conclusion on this point cannot be drawn from the available data.

Pituitary-induced diabetes and the metabolism of starvation. As the result of the classical investigations of Pettenkofer & Voit it is now generally accepted that, in starvation, protein and carbohydrate oxidation are curtailed and the onus of providing the major portion of the oxidative energy required for survival falls upon the stores of body fat. In this way the vitally important protein-containing tissues of the body are preserved from rapid destruction, the stores of glycogen in the muscles are protected from exhaustion and the blood sugar level is kept from falling more than a very little below that found during normal post-absorptive conditions. It is clear that the changes in metabolism which, as we have seen above, are associated with the administration of anterior pituitary extract to an intact animal receiving proteinaceous food are qualitatively similar to those which take place in a normal animal subjected to a period of starvation. Thus, if an animal which is consuming daily a normal ration of food is given each day a dose of suitable anterior pituitary extract, his basic metabolic pattern will resemble that of a starving animal despite his continued ingestion of a supply of food adequate to maintain a constant body weight under normal conditions. The oxidation of carbohydrate and protein being depressed at the expense of increased combustion of fat, the carbohydrate and protein stores of the body will be rapidly filled. If pancreatic activity is sufficient this process may result, under suitable conditions, in a sustained deposition of new tissue in the body, and growth may occur. If, on the other hand, the islets fail to provide adequate insulin, the protein (and carbohydrate) which cannot then be stored is partly eliminated as sugar (and urea) and partly burned. Such a reaction is dependent on the admission to the body of exogenous material and it is therefore understandable that the diabetogenic action of anterior pituitary extract cannot be demonstrated in a starving intact animal.

SUMMARY

1. In a normal intact animal (dog, cat, rat), consuming a daily ration of meat food only just sufficient to maintain a steady body weight under normal conditions, the daily injection of diabetogenic anterior pituitary extract led to nitrogen retention, a rise in body weight and, in the dog and cat, diabetes. Nitrogen retention continued throughout the period of diabetes even when the D/N quotient rose to 3·6 more (dog).

2. Analysis of muscle and liver (dog, cat) and of the whole carcass (rat) showed that much of the tissue added under pituitary influence must have possessed a composition similar to that of muscle. On this basis the retained nitrogen was more than sufficient to account quantitatively for the added tissue (dog, cat).

3. Despite the constancy of the food intake and the loss of energy by the deposition of new tissue (dog, cat, rat) and by the excretion of sugar (dog, cat), the metabolic rate of the pituitary-treated animals was apparently not depressed. Evidence was obtained that, in the pituitary-treated animal, the loss of calories, entailed by the formation of new tissue and by glycosuria, was more than made good by an increased rate of oxidation of stored fat.

4. An important action of the diabetogenic pituitary extract appears to be the partial replacement of carbohydrate and protein combustion by that of fat. The non-fasting animal treated with pituitary extract therefore resembles the untreated fasting animal in basic metabolic pattern.

5. Since the deposition of 1 g. of tissue possessing the composition of muscle results in an energy loss

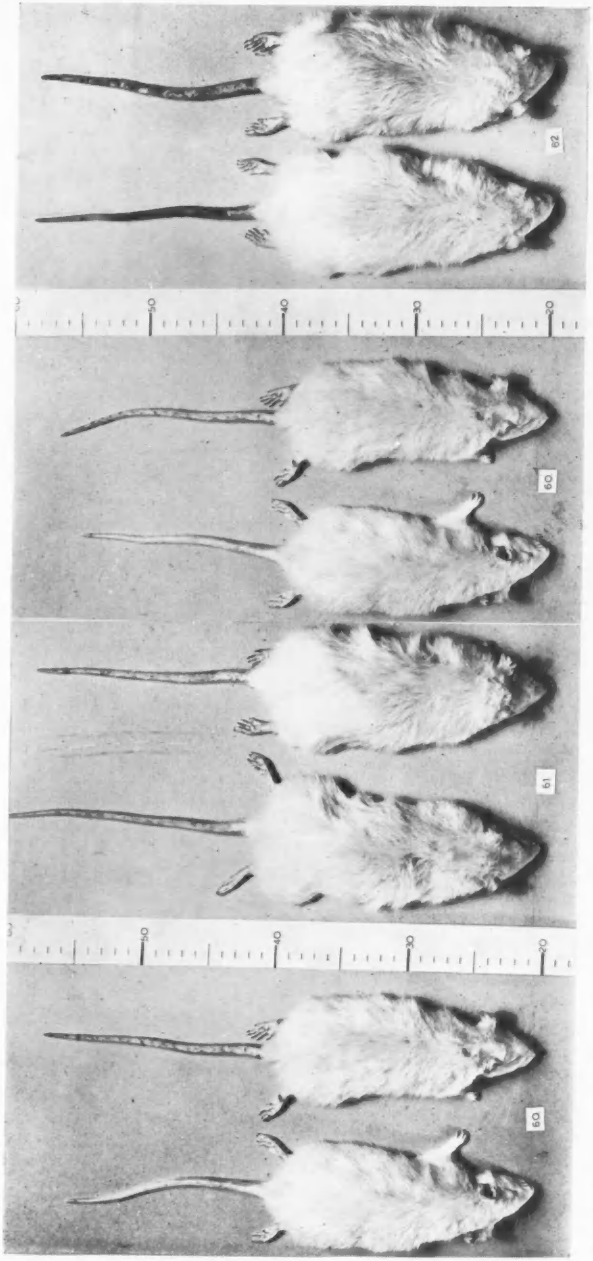

Fig. 6. Influence of daily injections of anterior pituitary extract over a period of 9 weeks in the body of the rat. The photographs are of representative pairs of rats from groups 60 (control injections—constant food), 61 (pituitary injections—constant food) and 62 (pituitary injections—unlimited food). The pairs of photographed animals were selected to have weights close to those of the mean for the group in question. The body weights were as follows:

Group	Body weight (g.) of photographed rats			Mean body weight (g.) of group
	Left	Right	Mean	
60	210	220	215	218
61	295	294	294	297
62	346	360	353	356

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of approximately 1 kg. cal., while the oxidation of 1 g. of depot fat liberates approximately 9 kg. cal., an equicaloric balance between the oxidation of extra depot fat and the deposition of tissue having the composition of muscle leads to a substantial rise in body weight. Such an effect accounts for much of the weight-increasing activity of the diabetogenic anterior-pituitary extract in an animal with constant food intake.

6. Despite the restriction of food intake and a substantial fall in body-fat content the pituitary-treated animals (dog, cat, rat) appeared to be plumper than their controls, and had a higher 'Nutritive Index' (rat). The pituitary treatment apparently resulted in a fall in body-fat content and a gain in protein-containing tissue and the mean specific gravity of the body substance rose. It is suggested that a knowledge of the mean specific gravity of the body might be helpful clinically in cases of pathological over-weight, particularly in that associated with diabetes mellitus.

7. The deposition of protein in the body requires the active co-operation of the pancreatic islets. In an animal susceptible to the diabetogenic action of anterior pituitary extract the islets probably pass through a phase of excessive insulin secretion, before they become relatively exhausted with the consequent development of diabetes. This secretory activity of the islets is believed to be an important factor in the nitrogen retention and growth promotion resulting from treatment with diabetogenic pituitary extract.

8. Diabetes probably represents the pathological result of an excessive stimulation of those processes (inhibition of oxidation of carbohydrate with enhancement of protein storage and of fat combustion) which, under more physiological conditions, lead to such deposition of new tissue as is associated with growth. In crude anterior pituitary extract the 'diabetogenic complex' may be identical with the complex agent responsible for the full growth-promoting activity of the extract.

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